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Lu, F., Kuhnle, G. K. ORCID: https://orcid.org/0000-0002-8081-8931 and Cheng, Q. ORCID: https://orcid.org/0000-0001-8198-8556 (2017) Vegetable oil as fat replacer inhibits formation of heterocyclic amines and 2 polycyclic aromatic hydrocarbons in reduced fat pork patties. Food Control, 81. pp. 113-125. ISSN 0956-7135 doi: 10.1016/j.foodcont.2017.05.043 Available at https://centaur.reading.ac.uk/70536/

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To link to this article DOI: http://dx.doi.org/10.1016/j.foodcont.2017.05.043

Publisher: Elsevier

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PII:	S0956-7135(17)30285-2
DOI:	10.1016/j.foodcont.2017.05.043
Reference:	JFCO 5649
To appear in:	*Food Control*
Received Date:	05 March 2017
Revised Date:	25 May 2017
Accepted Date:	26 May 2017

Please cite this article as: Fei Lu, Gunter K. Kuhnle, Qiaofen Cheng, Vegetable oil as fat replacer inhibits formation of heterocyclic amines and polycyclic aromatic hydrocarbons in reduced fat pork patties, *Food Control* (2017), doi: 10.1016/j.foodcont.2017.05.043

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ACCEPTED MANUSCRIPT

Vegetable oil as fat replacer inhibits formation of heterocyclic amines and polycyclic aromatic hydrocarbons in reduced fat pork patties

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Abstract

Formation of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) was examined to evaluate the impact of using vegetable oil as fat replacement on carcinogen formation in meat product. Pork patties were formulated with 40% fat replacement by olive oil, sunflower oil or grape seed oil, respectively and cooked at 180°C or 220°C. 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 (2-amino-3, 4-methylimidazo[4,5-f]quinoline), while grape seed oil completely inhibited MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) 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 reactions; Oxidation.

Chemical compounds studied in this article:

2-amino-3-methylimidazo[4,5-f]quinoline (PubChem CID: 53462); 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline (PubChem CID: 62274); 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (PubChem CID: 62275); 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (PubChem CID: 104739); 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PubChem CID: 1530); Benzo[a]pyrene (PubChem CID: 2336); Benz[a]anthracene (PubChem CID: 5954)

1. 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 (MUFA) and polyunsaturated fatty acids (PUFA) 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 MUFA in final products, whereas sunflower oil and grape seed oil could greatly raise the level of PUFA 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, Agregán, Gonçalves, and Lorenzo (2016) replaced 100% pork back fat with olive oil in pork pâté, which significantly increase the content of tocopherol and MUFA in cooked products without altering physio-chemical properties. Domínguez, Pateiro, Agregán, and Lorenzo (2017) and Lorenzo, Munekata, Pateiro, Campagnol, and Domínguez (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 PUFA 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 MUFA and PUFA.

However, unsaturated fatty acids in vegetable oils 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, 2012a; 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 heterocyclic amines (HCAs) (Morello, Shahidi & Ho, 2002; Turesky, 2010; Zamora & Hidalgo, 2007). Effect of fatty acids/oils on the formation of HCAs has been

documented in previous research. Johansson et al. (1995) reported that the higher level of MeIQx and DiMeIQx were found in burgers fried in rapeseed oil containing high level of oleic acid with high peroxides values, compared with butter, margarine and sunflower oil. 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 accelerate the formation of PhIP in chemical model system. Some hydroperoxides generated from the decomposition of the unsaturated hydrocarbons during heating, such as linolenate acid can also undertake aromatization and de-hydrocyclization, further cleave into benzaldehydes and other benzene ring-containing compounds, which are precursors of polycyclic aromatic hydrocarbons (PAHs) (Chen & Chen, 2001; Lorenzo et al., 2011; Lorenzo, Purriños, Fontán, & Franco, 2010; 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

98 smoking meat products likely contain high level of PAHs (Alomirah et al., 2011).
99 Benz[a]anthracene (BaA) and benzo[a]pyrene (BaP) are the most potent
100 carcinogenic PAHs in processed meat products (PHE, 2008). The metabolite of BaP,
101 BaP-7,8-diol-9,10-epoxide, has been reported with the highest tumour-inducing
102 activity due to causing DNA adducts (Purcaro, Moret & Conte, 2013).

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

118 **2. Material and methods**

119 **2.1 Materials**

120 Three batches of lean pork leg and pork back fat with 40.3% SFA, 43.4% MUFA and
121 10.0% PUFA (McCance & Widdowson, 2002) were purchased from Jennings
122 Caversham (Reading, UK) at different time point to consider the batch effect.

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 -18 ^{o}C and defrosted 24h at 4 ^{o}C 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 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 100-300mg tocopherols (McCance & Widdowson, 2002) were purchased from local supermarket (Reading, UK). Oils were kept in refrigerator (4^{o}C) before making patties and further analysis.

The 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), 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), 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-

148 2,5,7,8-tetramethylchromane-2-carboxylic acid (*Trolox*), phosphate buffer solution
149 0.1 M and phosphoric acid were purchased from *Sigma-Aldrich* (Gillingham, UK).
150 The solid-phase extraction *Extrelut NT 20* columns and diatomaceous earth refill
151 material were purchased from *Merck* (Darmstadt, Germany). Bond Elut propyl-
152 sulfonic acid (PRS) cartridges (100 mg, 10 ml), C-18 cartridges (7 ml) were
153 purchased from *VWR Inc* (Lutterworth, UK).

154 **2.2 Procedures for preparing and cooking pork patties**

155 The formulation of control patties (as shown in Table 2) included 700g lean pork
156 mince, 180g distill water, 100g pork back fat and 20g salt per kilogram. For the fat
157 partially replaced patties, 40% of pork back fat was replaced with sunflower oil, olive
158 oil or grape seed oil respectively. Overall, there were 4 types of pork patties
159 prepared in this study, i.e. control (100% back fat, C patties), sunflower oil patties (S
160 patties), olive oil patties (O patties) and grape seed oil patties (G patties). All
161 ingredients were homogenized at 5000rpm for 5min in the Kenwood Food processor
162 to reach a uniform emulsion. Each patty was weighed 100g, shaped in a foil cup
163 (9.0cm diameter * 2.5cm height) for standardization.

164 Patties were cooked in an air-forced oven at either 180 ^{o}C or 220 ^{o}C. Cooking was
165 terminated when core temperature of patties reached to 73 ^{o}C. After cooking, pork
166 patties were covered by foil and chilled in cold room at 4 ^{o}C for 24 hours. Physical
167 properties including texture and colour were measured on the following day of
168 cooling. While part of samples were homogenized and stored in -18 ^{o}C for further
169 chemical analysis. Cooking loss was determined according to the equation: Cooking
170 loss (%) = $(W_r - W_c)/W_r$, where W_r was the weight of raw pork patties, and W_c was

171 weight of cooked pork patties. All the treatments were replicated three times. For
172 each replicate, 8 patties were made for each treatment.

173 **2.3 Composition analysis**: **pH, moisture, fat and protein content**

174 pH was measured by inserting a pH meter (68X243601, Oakton Instruments, USA)
175 into the mixture by blending 5g meat sample with 45ml distill water (Puangsombat et
176 al., 2011). The moisture content was determined by drying 3g meat samples in an
177 oven at 100^{o}C for 24 hours according to AOAC methods (Horwitz & Latimer, 2005).
178 The fat content was determined by drying sample in an oven for 4 hours firstly then
179 using Soxhlet extraction system. The protein content was determined using the
180 Kjeldahl method (Horwitz & Latimer, 2005).

181 **2.4 Lipid/protein oxidation and antioxidant capacity**

182 **2.4.1 Lipid oxidation--Thiobarbituric acid-reactive substances (TBARS) value**

183 The degree of lipid oxidation in samples was expressed by TBARS values, which
184 were determined by the method reported by Rodríguez-Carpena et al. (2012). 5 g
185 well blended pork patty was homogenized with 15 ml perchloric acid (3.86%) and
186 0.5ml BHT (4.2% in ethanol) in a beaker, which was immersed in an ice bath to
187 minimize oxidative reactions in samples during extraction. The mixture was then
188 filtered and centrifuged at 3000 rpm for 4 min, 2 ml supernatant was mixed with 2 ml
189 thiobarbituric acid (0.02 M) in test tube. The test tubes were then placed in a boiling
190 water bath (100 ^{o}C) for 45 min. After cooling, the absorbance was measured at
191 532nm using a spectrophotometer (6315, Bibby Scientific Ltd, UK). The standard
192 curve was prepared using 1,1,3,3-tetraethoxypropane (TEP) in 3.86% perchloric acid
193 with the concentration of 0, 0.5, 1.0, 2.5, 5.0 and 10.0μM.

2.4.2 Total protein carbonyl value (Protein oxidation)

The degree of protein oxidation can be evaluated by calculating the total carbonyl value according to the method described by Rodríguez-Carpena et al. (2012). 1g of well blended pork patties was homogenized at 1:10 (w/v) 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 2 M HCl in order to measure protein concentration, while the other pellet was mixed with 1 ml of 0.2% (w/v) dinitrophenylhydrazine (DNPH) in 2 M 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 6 M guanidine HCl (pH 6.5), stirred and centrifuged for 2 min at 5000 rpm to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using BSA as standard. The amount of carbonyls was expressed as nmol of carbonyl per mg of protein using an absorption coefficient of 21.0 nM^{-1} cm^{-1} for absorbance at 370 nm for DNPH.

2.4.3 *Trolox* equivalent antioxidant capacity (TEAC) of vegetable oil/ 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 2.5 mM ABAP with 20 mM $ABTS^{2-}$ stock solution in 100 mM phosphate buffer (pH 7.4), which

contained 150 mM NaCl. The solution was covered with foil and heated at 60°C for 12 min, then cooled down to room temperature. 40 µl of the sample solution was mixed with 1960 µl of the freshly prepared ABTS/ABAP 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 oil or back fat was presented on a molar basis to *Trolox* (µmol Trolox/100g).

2.5 Analysis of HCAs

HCAs extraction and purification were following the procedures reported 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 with sauce before measuring. 3g ground meat sample was mixed with 12ml 1M sodium hydroxide firstly, then the mixture was 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 2ml vial, followed by drying the mixture under nitrogen stream for 1.5h at room temperature. The residue in the vial was dissolved with 1ml methanol and submitted for HPLC analysis. 1ml of mixtures containing 5 standard compounds (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx

and PhIP) (5 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°C. The UV detector was set at 252 nm (Puangsombat et al., 2011).

2.6 Analysis 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 column*, and was followed by propyl sulphonic acid (PRS) cartridges with 60 ml of CH_2Cl_2 containing 5% toluene. The dichloromethane solution was then evaporated to small amount (0.5-1ml) and the rest of the solvent was dried under 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 25 ml of n-hexane and 60 ml of 60:40 (v/v) n-hexane–CH_2Cl_2 mixtures. After evaporation to dryness the residues were dissolved in acetonitrile (spiked and unspiked samples) before the HPLC analysis (Janoszka, 2011). 50ng of 2 standard mixtures (BaA and BaP) 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 conditions. The separations were performed at 40^{o}C under isocratic conditions 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).

2.7 Recovery rate of HCAs and PAHs

The 5 standard HCA (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 the quantity of each individual compound was determined according to the standard calibration curves, which was established by the standard solution at 0.5ng/ml, 5ng/ml and 50ng/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, Jinap, Abas, & Sakar, 2010; Ishizaki, Saito, Hanioka, Narimatsu, & Kataoka, 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, Heitz, Cuomo, Badoer,

& Traverso, 2006). Stevens, Hamstra, Hegeman & Scharrer (2006) reported that overlapping peaks on chromatogram caused by 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.

2.8 Inhibitory rate of HCAs and PAHs

Inhibitory rate was determined according to the equation:

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

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

2.9 Physical parameters

2.9.1 Colour

Colour feature including L*, a* and b* was measured using Hunter Lab Colour instructment (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.

2.9.2 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

313 calculation of TPA values was based on the compression curve with force (y-axis)
314 and time (x-axis). Values for hardness (N) was defined as the absolute peak force in
315 the 1st compression cycle, cohesiveness as the area of work in the 2nd compression
316 divided by the area of work in the 1st compression, chewiness as the product of
317 hardness*cohesiveness*springiness, and springiness as the force that sample return
318 to its initial pattern after compression (Sánchez del Pulgar, Gázquez & Ruiz-
319 Carrascal, 2012). Each sample was measured at least 5 times, and the average was
320 recorded as the value of the sample.

321 **2.10 Statistical analysis**

322 Each treatment had three replicates. Statistical significance test was carried out by
323 using SPSS Statistics 21 (IBM, 2014). The significant difference in chemical
324 composition, physical property, levels of HCAs and PAHs for the 8 treatments were
325 carried out by one-way analysis of variance (ANOVA) at the significant level 0.05,
326 and *Duncan* test was selected for multiple comparison if equal variances assumed,
327 otherwise *Tamhane's T2* test was used. To analyse the effect of factors and the
328 interaction between factors (cooking temperature and replacing oil type), two-way
329 ANOVA was employed at the significant level 0.05. Multivariate linear regression
330 model was employed to explore the effect of multi independent factors, including lipid
331 oxidation, protein oxidation and antioxidant capacity of oils on the formation of HCAs
332 and PAHs at the significant level 0.05. Pearson correlation was employed for the
333 measurements as a prerequisite for the regression.

334 **3. Results and discussion**

335 **3.1 Effect of replacing oil and cooking temperature on proximate composition**
336 **and physical parameters of reduced fat pork patties**

3.1.1 Proximate composition

Table 2 shows that 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 $63.36 \pm 0.37\%$ to $67.53 \pm 0.26\%$ 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. There was no significant difference in moisture content for all the patties (with or without fat replacement) ($p=0.206$), 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 ranging 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 °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 these 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 ($p>0.05$).

3.1.2 Physical parameters

3.1.2.1 Texture

Texture property of cooked pork patties was examined including hardness, cohesiveness, springiness and chewiness through a typical texture profile analysis. Results were listed in Table 3. Cooking temperature affected hardness, cohesiveness and chewiness ($p \leq 0.05$), but had no effect on springiness ($p>0.05$). 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, chewiness and springiness except cohesiveness ($p>0.05$).

High cooking temperature led to high hardness. Control patties cooked at 220°C had significantly higher hardness (26.65±3.15N) than that cooked at 180°C (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 that partially (50%) replaced with sunflower oil, olive oil and avocado oil had same hardness with control patties. However, Hur, Jin and Kim (2008) reported that olive oil replacement

385 in pork patties resulted in low hardness compared with control patties. The cooking
386 temperature could help to explain the disagreement. In their study, patties were
387 cooked at 100 ^{o}C in water bath, while samples were cooked in convection oven at
388 180^{o}C or 220^{o}C. The hardening effect of high cooking temperature could be
389 neutralized by the softening effect of to the replacing vegetable oil. As a result, there
390 was no difference observed in hardness for fat modified patties and control sample
391 ($p>0.05$).

392 Temperature affected cohesiveness greatly ($p=0.001$), because the texture
393 parameters are mainly determined by denaturation of the structural protein system,
394 i.e. actomyosin complex and collagen (Palka & Daun, 1999). Type of oil did not have
395 any effect on the cohesiveness of pork patties ($p>0.05$). Rodríguez-Carpena et al.
396 (2012) also reported that there was no difference in cohesiveness between control
397 and patties with 50% fat substitution using avocado, sunflower or olive oil. The
398 interaction between temperature and type of oil on cohesiveness was observed
399 ($p<0.05$). For control, olive and sunflower oil treatment, patties cooked at 220^{o}C had
400 higher cohesiveness than these cooked at 180^{o}C ($p<0.05$), but there was no
401 difference in cohesiveness for grape seed oil samples cooked at different
402 temperatures ($p>0.05$). This interaction between cooking temprature and type of oil
403 could be explained by the emulsion stability of oil/fat emulsion. Youssef and Barbut
404 (2009) and Rodr´ıguez-Carpena et al. (2011) reported that vegetable oil with high
405 polyunsaturated fatty acids (PUFA) had small fat globules in meat emulsion, which
406 could offer a stronger fat-protein interaction. Grape seed oil contained high level of
407 PUFA compared with back fat and olive oil, which led to a stable emulsion in G
408 patties. In addition, polyphenol compounds in grape seed oil emulsion could help
409 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±0.77 N.s in C patties, and 5.35±0.41 N.s in S patties that cooked at 220 °C. Greater hardness, cohesiveness and chewiness at higher elevating 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 2). Therefore, high chewiness would be expected in samples cooked at 220°C due to high moisture loss.

3.1.2.2 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. 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 that 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).

3.2 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 4. Type of

458 oil affected all individual HCAs compound except IQ, cooking temperature
459 significantly affected the total amount of HCAs and all individual HCAs compounds
460 except MeIQx. Interaction between oil and cooking temperature was observed in
461 total HCAs, IQ, MeIQ, 4, 8-DiMeIQx and PhIP, but not MeIQx. At both temperatures,
462 all fat modified patties had significantly lower amount of MeIQ, 4, 8-DiMeIQx and
463 total HCAs than control patties ($p<0.05$). MeIQx, 4, 8- DiMeIQx and PhIP were not
464 detectable in G patties. Tocopherols (average 50mg/100g in sunflower oil, 100-
465 300mg/g in refined olive oil and 10-15mg/g in grape seed oil) and polyphenols
466 (common profile in grape seed oil: catechin, epicatechin and epicatechin gallate) in
467 these oils could play roles in reducing the final HCAs in patties (Bail et al., 2008;
468 McCance & Widdowson, 2002; Rombaut et al., 2014). Tocopherols have been found
469 to block dialkyl-pyrazine radicals for further reaction with creatine to form HCAs, or
470 react with precursors of 4, 8-DiMeIQx to inhibit the formation of HCAs (Pearson et al.,
471 1992; Vitaglione & Fogliano, 2004). Phenolic compounds could also prevent the
472 formation of imidazoquinoxaline-type HCAs through trapping pyrazine cation radicals
473 and some other carbon-centred radicals generated either from pyrazine cation
474 radicals or different pathway during Maillard reaction (Kato et al., 1996). In addition,
475 phenolic compounds have the ability to directly trap phenylacetaldehyde, which is a
476 major intermediate during the formation of PhIP (Cheng et al., 2007).

477 Total HCAs ranged from not detected (Nd) to 140.57 ± 22.03 ng/g. Control patties
478 cooked at both cooking temperatures contained significantly higher amount of total
479 HCAs (67.56 ± 17.29 ng/g and 140.57 ± 22.03 ng/g), followed by S patties ($5.98 \pm$
480 1.10 ng/g and 23.88 ± 2.44 ng/g) and O patties (4.11 ± 0.87ng/g and 20.03 ± 2.25
481 ng/g), while G patties achieved the lowest total HCAs in both temperatures (Nd and
482 1.90 ± 0.04 ng/g). Control samples cooked at 220°C contained all types of HCAs,

483 whereas none of HCA compounds were detected in G patties cooked at 180°C. The
484 dominating compounds of HCAs were MeIQ (59.70 ± 0.98ng/g) and 4, 8-DiMeIQx
485 (43.37 ± 15.67ng/g) in C patties, while PhIP in S (14.78 ± 1.49ng/g) and O patties
486 (22.70 ± 1.95ng/g). The total HCAs in C patties were higher than some published
487 results. Vangnai et al. (2014) reported MeIQx (7.59 ± 0.43ng/g), PhIP (13.12 ± 0.72
488 ng/g) and total HCAs (22.35 ± 1.17 ng/g) in fried pork loins cooked at 204 °C for 16
489 minutes. The total level of HCAs in pan-fried well-done pork was 49.7ng/g with
490 cooking ended at 80 °C core temperature (Iwasaki et al., 2010). The sampling
491 procedure for measuring HCAs could help explain the difference. HCAs were
492 extracted from the 2mm outer layer surface of samples in this study, while lots of
493 researchers extracted HCAs from entirely ground samples. The precursors of HCAs,
494 such as creatine, glucose and free amino acids would migrate to the surface of meat
495 and enhance Maillard reactions during cooking (Gibis & Weiss, 2015). As a result,
496 surface could accumulate much higher level of HCAs compared with internal part of
497 the sample. Therefore, a higher concentration of HCAs would be expected than that
498 exacted from entirely ground samples.

499 IQ was detected up to 3.88 ng/g in cooked patties, which was in the range of 0.7-5.3
500 ng/g in fried ground beef patties (Balogh et al., 2000). At 180 °C, IQ was not detected
501 apart from O patties, but cooking at 220°C generated high level of IQ in all patties
502 ($p<0.05$). Different vegetable oils did not affect the formation of IQ ($p>0.05$), but
503 interaction between vegetable oil and temperature was observed in formation of IQ.
504 IQ is generally formed through reactions between creatinine, pyridine radicals and
505 formaldehydes (Vitaglione & Fogliano, 2004). Vegetable oils could decompose into
506 hydroperoxides, and then aldehydes and ketones at high cooking temperature,
507 which further react with amino acids in Maillard reactions (Johansson, Skog, &

508 Jagerstad, 1993; Zamora & Hidalgo, 2007). Olive oils with high level of oleic acid
509 could decompose into aldehydes (-CHO) much faster (3-15 times) than sunflower oil
510 and grape seed oil containing high amount of linoleic and linolenic acid at 190°C
511 (Guillén & Uriarte, 2012b). This might explain why IQ was only detected in olive oil
512 patties at 180°C. But the pathway of formation of HCAs in the real meat system was
513 more complicated than chemical model system, and antioxidants in oils should also
514 be considered (Johansson, Skog, & Jagerstad, 1993). Therefore, further
515 investigation is needed to understand promoting mechanism and role of antioxidants
516 in oils. The highest MeIQ was found in control patties cooked at 220 °C (59.70±0.98
517 ng/g), followed by in control patties cooked at 180 °C (18.26±14.46 ng/g). Janoszka
518 (2010) reported that 6.28 ng/g MeIQ was detected in pan-fried pork patties cooked at
519 170°C for 12 minutes, which was similar with this study. Formation of MeIQ was
520 completely inhibited by olive oil and sunflower oil at both cooking temperatures.
521 Grape seed oil could only inhibit formation of MeIQ at low cooking temperature, while
522 1.31ng/g was detected in G patties cooked at 220°C. The inhibitory effect on MeIQ
523 could be attributed to the antioxidants such as vitamin E and polyphenols in
524 vegetable oils. Rounds et al. (2012) and Liao et al. (2009) also reported that vitamin
525 E and polyphenols could reduce the formation of MeIQ. Balogh et al. (2000) found
526 that vitamin E had stronger inhibitory effect on MeIQ with reduction rate 64.3% than
527 phenolic compound in Oleoresin rosemary extract with reduction rate 47.9% in fried
528 beef patties. Since olive oil and sunflower oil contain higher level of vitamin E than
529 grape seed oil, therefore, stronger inhibition of MeIQ would be expected in O patties
530 and S patties. Cooking temperature did not affect MeIQx level in patties ($p>0.05$), but
531 formation of MeIQx was significantly affected by different vegetable oils. S patties
532 and O patties had similar MeIQx with control sample. 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 oil 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 $\pm$ 11.5 ng/g) in the 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 phenolic 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 175-200 °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 °C.

Type of vegetable oil significantly affected the level of total HCAs in cooked patties ($p<0.05$), but temperature did not have any effect ($p>0.05$). Interaction between types of vegetable oil and cooking temperature was observed as well ($p<0.05$). 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 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 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 1). Therefore, reduction of HCAs in vegetable oils, especially grape seeds oil was expected compared with control sample. 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 reactions. 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. Phenolic compounds,

583 such as catechin, epicatechin-3-O-gallate, oligomer procyanidins and tocopherols in
584 grape seed oil contributed to its antioxidant capacity (Agostini et al., 2012; Crews et
585 al., 2006; Matthäus, 2008). Vitaglione and Fogliano (2004) suggested that mixture of
586 antioxidant compounds could perform better than single antioxidant as they could
587 inhibit various pathways in different steps of reactions. Therefore, phenolic
588 compounds might work synergistically with tocopherols to enhance each other to
589 inhibit the formation of HCAs. However, the synergistic effect between different
590 antioxidants needs to be further examined.

591 Cooking temperature significantly affected total HCAs in pork patties ($p<0.01$) (Table
592 4). Patties cooked at 220^{o}C had significantly higher level of toal HCAs than these at
593 180^{o}C ($p<0.01$). Effect of temperature on the formation of HCAs was well examined
594 in previous research (Knize et al., 1994; Liao et al., 2009; Oz & Kaya, 2011).
595 Thermal processing has vital influence on the formation of polar HCAs (IQ, MeIQ,
596 MeIQx, DiMeIQx and PhIP), which are formed in meat products when samples are
597 cooked at 160-250 ^{o}C, typical domestically cooking temperature. High cooking
598 temperature generated more diverse types of HCAs, but also stimulate the
599 accumulation of the amount of HCAs on the surface of meat products (Olsson &
600 Pickova, 2005; Skog, Johansson & Jaègerstad, 1998).

601 **3.3 Effects of vegetable oils and cooking temperature on the formation of**
602 **PAHs**

603 Concentration of PAHs (BaA and BaP) in cooked pork patties with different cooking
604 temperature was listed in Table 5. The range of total PAHs was from1.59±0.26 ng/g
605 to 3.84±0.21 ng/g. The dominating compound of PAHs was BaP in all samples. BaA
606 ranged from 0.14-0.31 ng/g in cooked patties, while BaP ranged from 1.44 to

607 3.53ng/g. Temperature did not affect the formation of both BaA and BaP, but type of
608 vegetable oil had significant effect on the formation ($p<0.05$). Interaction between
609 type of oil and cooking temperature was also observed in both compounds (Table 5).
610 BaP level in this study (1.44-3.53 ng/g) are consistent with results reported by Nisha
611 et al. (2015) and Janoszka (2011), i.e. 1.52 ng/g of BaP in the oven broiled pork and
612 1.61 ng/g BaP in oven grilled pork chop (17min at 170^{o}C). Olive oil and grape seed
613 oil showed inhibitory effect on BaP when patties cooked at 220 ^{o}C, but no effect or
614 even promoting effect was observed at 180 ^{o}C. On the contrast, sunflower oil offered
615 inhibition on BaP at 180 ^{o}C, but promotion at 220 ^{o}C. As BaP is one of the highest
616 toxic potency compounds during meat cooking, EU Commission has regulated that
617 the updated limit of BaP occurring in processed meat and seafood products is 2 ng/g
618 (Purcaro et al., 2013; Wretling et al., 2010). Among all the patties, only O patties
619 cooked at 220 ^{o}C and S patties cooked at 180 ^{o}C met the safety regulation of BaP.
620 Therefore, it is necessary to develop any procedures or alternative methods that
621 reduce the amount of BaP to safety limit.

622 The effect of oil and interaction between type of oil and cooking temperature on the
623 formation of PAHs were significant ($p<0.05$). Cooking temperature did not affect the
624 formation of PAHs ($p>0.05$). S patties cooked at 220 ^{o}C had the highest total PAHs
625 (3.84±0.21 ng/g), followed by G patties (3.46±0.16 ng/g) cooked at 180 ^{o}C and C
626 patties (3.28 ± 0.07 ng/g) cooked at 220 ^{o}C. O patties cooked at 220^{o}C obtained the
627 lowest PAHs. PAHs were mainly associated with the pyrolysis of fat undertaken at
628 high temperature (Viegas et al., 2012). Therefore, smoking point of vegetable oils
629 may help explain the difference in PAHs. Sunflower oil and grape seed oil contain
630 high content of PUFAs, especially linolenic acid and linolenic acid have lower smoke
631 points (grape seed oil 216^{o}C, sunflower oil 227^{o}C) which makes them easy to

632 decompose, compared with olive oil (smoke point 242^{o}C). The decomposition of oil
633 could generate more reactive free radicals to accelerate the production of PAHs
634 (Chen & Lin, 1997; Elmore et al., 2002). They also concluded that hydroperoxides
635 from lipid oxidation, could subsequently generate cyclic compounds through
636 intramolecular reaction, and PUFA could undergo further polymerization. In addition,
637 vegetable oils themselves contained BaP, which might increase the total amount of
638 PAHs in cooked meat. Fromberg, Højgard and Duedahl-olesen (2007) reported that
639 olive oil, sunflower oil, and grape seed oil approximately contained 0.12 ng/g, 0.4
640 ng/g and 1.0 ng/g BaP respectively. As a result, high level of PAHs was expected in
641 sunflower oil and grape seed oil samples. Although vegetable oils contain
642 antioxidants, the inhibitory effect on PAHs formation was not observed consistently.
643 In S patties, the inhibitory efficiency at both temperature were 7.75% and 51.52%,
644 but olive oil and grape seed oil increased the formation of PAHs by 17.07% and
645 34.11%, respectively. It shows that antioxidants in vegetable oils were not involved in
646 the formation of PAHs to a great extent, which is further confirmed by the correlation
647 analysis. As indicated in Table 6, there is no correlation relationship observed
648 between antioxidant capacity of oil (TEAC) and total PAHs. The impact of
649 tocopherols and phenolic compounds on the formation of PAHs in processed meat
650 were not well documented. In vitro study, Zhu et al. (2014) found that vitamin E
651 intake could significantly prohibit free radicle induced by BaP and protect cellular
652 damage in human lung, but the effect antioxidants on formation of PAHs in food
653 products has been scarce.

654 **3.4 Correlation between lipid oxidation, protein oxidation and the formation of**
655 **HCAs and PAHs**

In Figure 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 phenolic compounds) within the oils, since a significantly negative relationship was found between TBARS values and antioxidant capacity of oils ($r= -0.764$, $p<0.01$). Wong et al. (2015) reported that 0.1-0.4mmol 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) oligomer procyanidins were also sufficient to inhibit lipid oxidation by reducing free radicles 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 in the formation of HCAs.

Protein carbonyls are produced from protein oxidative degradation in meat products, which were used to analyse degree of protein oxidation (Figure 3). Significant effect

681 of vegetable oils on the protein oxidation was observed ($p=0.001$). C patties had a
682 significantly higher level of protein carbonyls (12.11 mmol/kg) than other 3 fat
683 modified patties ($p<0.05$). The protein oxidation could be inhibited by the presence of
684 antioxidants in oil, as negative correlation between antioxidant capacity of oils (TEAC)
685 and protein carbonyl level was found with $r=-0.606$, $p<0.01$, as indicated in Table 6.
686 Botsoglou et al. (2014) found that protein carbonyl value could be reduced from 3.25
687 mmol/kg to 2.25 mmol/kg in cooked pork patties when 50mg/kg α-tocopherol was
688 added. Vuorela et al. (2005) reported that phenolic compounds, including
689 vinylsyringol and sinapic acid in rapeseed oil had good antioxidant capacity against
690 protein oxidation in cooked pork patties. Ganhão et al. (2010) also found that
691 arbutus-berries extract containing catechins significantly reduced protein oxidation
692 by chelating heme iron in cooked patties. However, there was no difference in the
693 protein carbonyl level among O, S and G patties ($p>0.05$).

694 In the development of HCAs under high temperature cooking, both lipid oxidation
695 and protein oxidation are involved with Maillard reactions in the meat system
696 (Johansson, Skog & Jagerstad, 1993; Zamora & Hidalgo, 2007). Lot of researchers
697 believed that formation of HCAs could be primarily related to interactions between
698 free radicals generated from lipid oxidation and free radicals produced in Maillard
699 reactions (Hwang & Ngadi, 2002; Skog et al., 1998). Therefore, it is useful to explore
700 the relationship between lipid/protein oxidation and the formation of HCAs. In this
701 study, correlation analysis was conducted between TBARS/protein carbonyl values
702 and concentration of total HCAs in fat modified cooked patties (Table 6). Significant
703 positive correlation was disclosed between total HCAs and TBARS ($r=0.826$, $p<0.01$)
704 and between HCAs and protein carbonyl ($r=0.778$, $p<0.01$), which further confirmed
705 that both lipid oxidation and protein oxidation participated the formation of HCAs

706 during cooking process. In order to further examine the relationship between lipid
707 oxidation/protein oxidation/antioxidant capacity of lipids and total HCAs in cooked
708 patties, multivariate linear regression model was displayed below,

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

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

719 Free radicals, such as aldehydes and ketones generated from lipids oxidation could
720 interact with Maillard reactions by reacting with the polar head of an amino group to
721 produce more HCAs (Jägerstad et al., 1998; Zamora & Hidalgo, 2007). On the other
722 hand, active protein carbonyl residues, such as alkyl, peroxyl radicals that formed by
723 muscle protein oxidation can be initialized by lipid oxidation, metal ions and other
724 peroxided compounds (Cai et al., 2002). Subsequently, these carbonyls could
725 interact with Maillard reaction via Schiff base and then generate Strecker aldehydes,
726 which are intermediates of imidazoquinolines and imidazoquinoxalines (Estévez,
727 2011; Soladoye et al., 2015). Researchers also suggested that lipid oxidation could
728 trigger protein oxidation by reacting with heme iron that released from myoglobin
729 (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 ($p>0.05$).

4. Conclusions

Control patties contained the highest amount of HCAs and relatively higher PAHs at 180 ^{o}C and 220 ^{o}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 inhibitive 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. Due to the synergistic effect of antioxidants, it would be interesting to explore replacing pork back fat with a mixture of several types of oils to reach an optimum fatty acids profile,

instead of replacing pork back fat with one single type of oil. Overall, replacing pork back fat with vegetable oils in processed meat products could offer healthier meat products with reduced HCAs without compromising eating quality.

Acknowledgement

We thank C. Humphrey, C. Busey, M. Puranik and other technicians at University of Reading for the technical support.

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1127 Table 1: Formulation of control and fat reduced 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

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1135 Table 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

1136 [a] Results with different letters in the same column are significantly different at the level p<0.05.
1137 [b] Values represented as the Mean ± standard deviation (SD), n=3.

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Table 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)	Springiness	L*	a*	b*
Control	180	20.14±2.81^{a}	0.34±0.02^{ab}	4.06±0.77^{ab}	0.71±0.03^{a}	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}	0.77±0.04^{a}	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}	0.72±0.04^{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}	0.75±0.02^{a}	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}	0.73±0.05^{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}	0.74±0.03^{a}	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}	0.77±0.01^{a}	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}	0.77±0.04^{a}	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	0.341	0.062	0.54	0.65
p-value (Temperature)		0.05	0.001	0.001	0.085	0.05	0.005	0.05
p-value (Interaction between oil*temperature)		0.41	0.02	0.33	0.36	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.

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1142 Table 4: Heterocyclic amines in cooked pork patties with partial replacement of fat by vegetable oils at 180 °C and 220 °C [a,b,c]

Treatment	Cooking temperature	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	-

1143 [a] Results with different letters in the same column are significantly different at the level p<0.05.
1144 [b] Values represented as the Mean ± SD, n=3.
1145 [c] Nd: Not Detected.
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Table 5: Polycyclic aromatic hydrocarbons 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.

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1152 Table 6: Pearson correlation coefficient (p) 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

1153 ** Significant level 0.01
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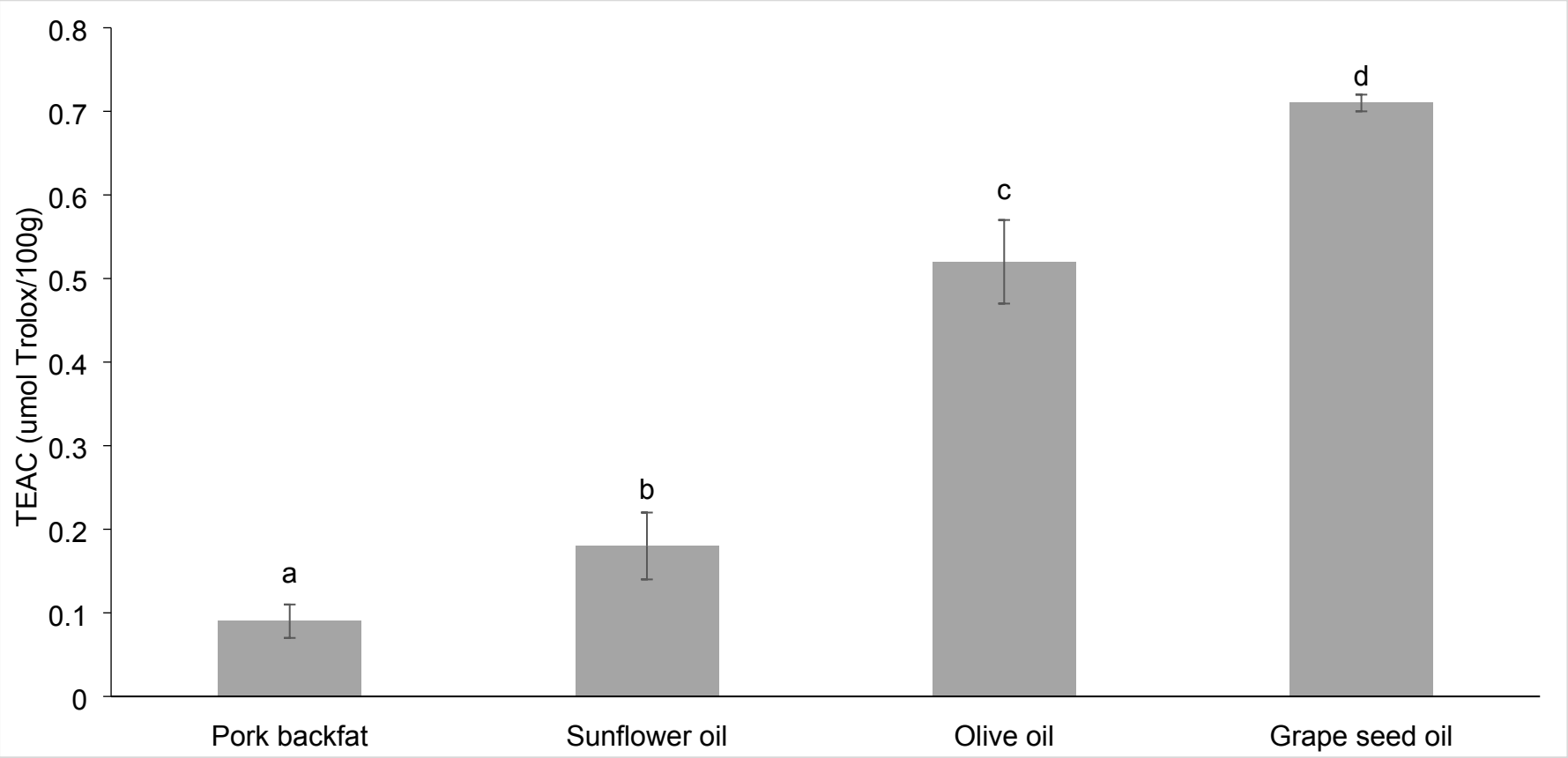
Figure 1: Trolox Equivalent Antioxidant capacity (umol Trolox/100g) of pork backfat and 3 vegetable oils.



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

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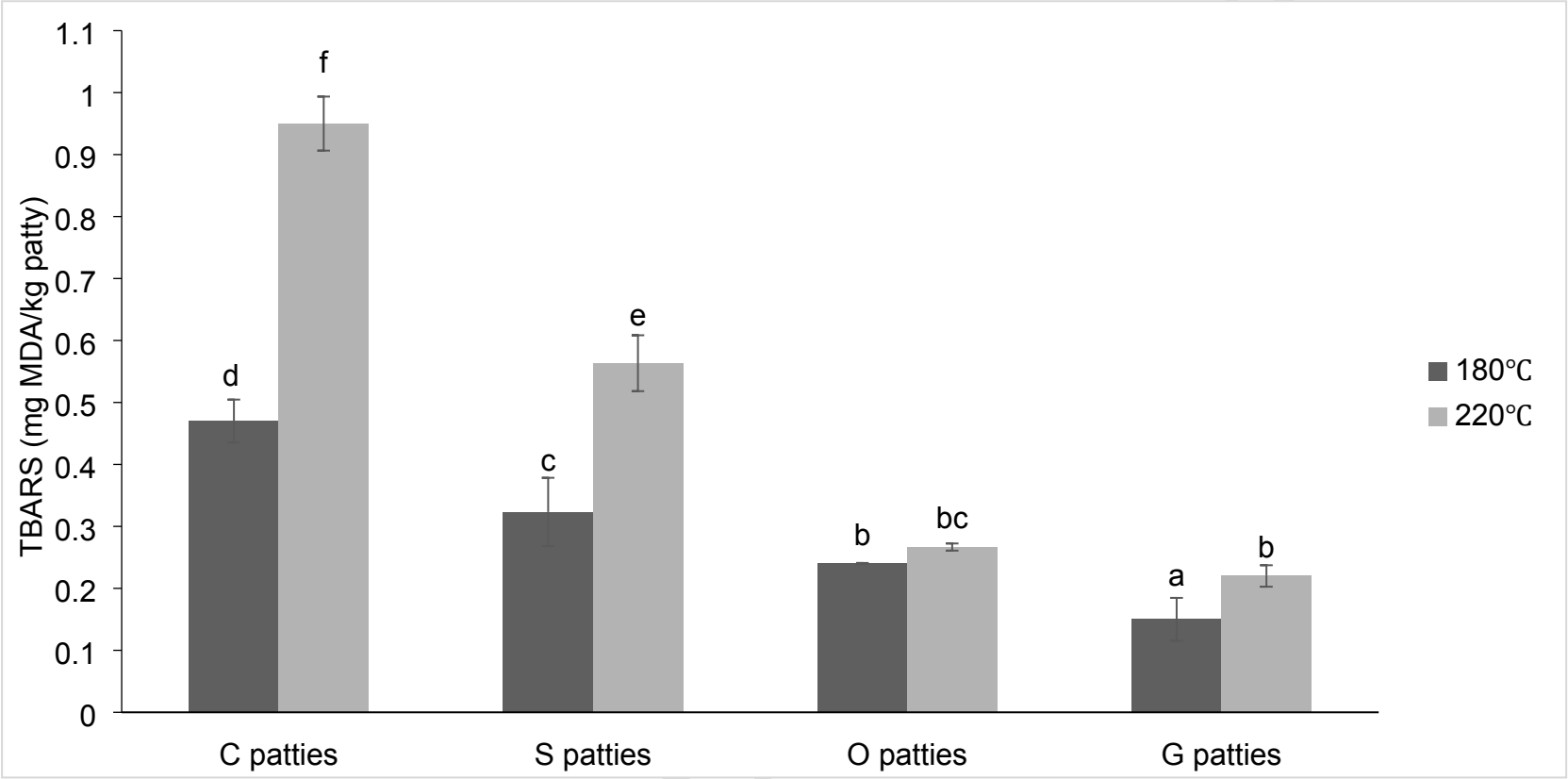
Figure 2: TBARS values in pork patties cooked at 180 °C and 220 °C.



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

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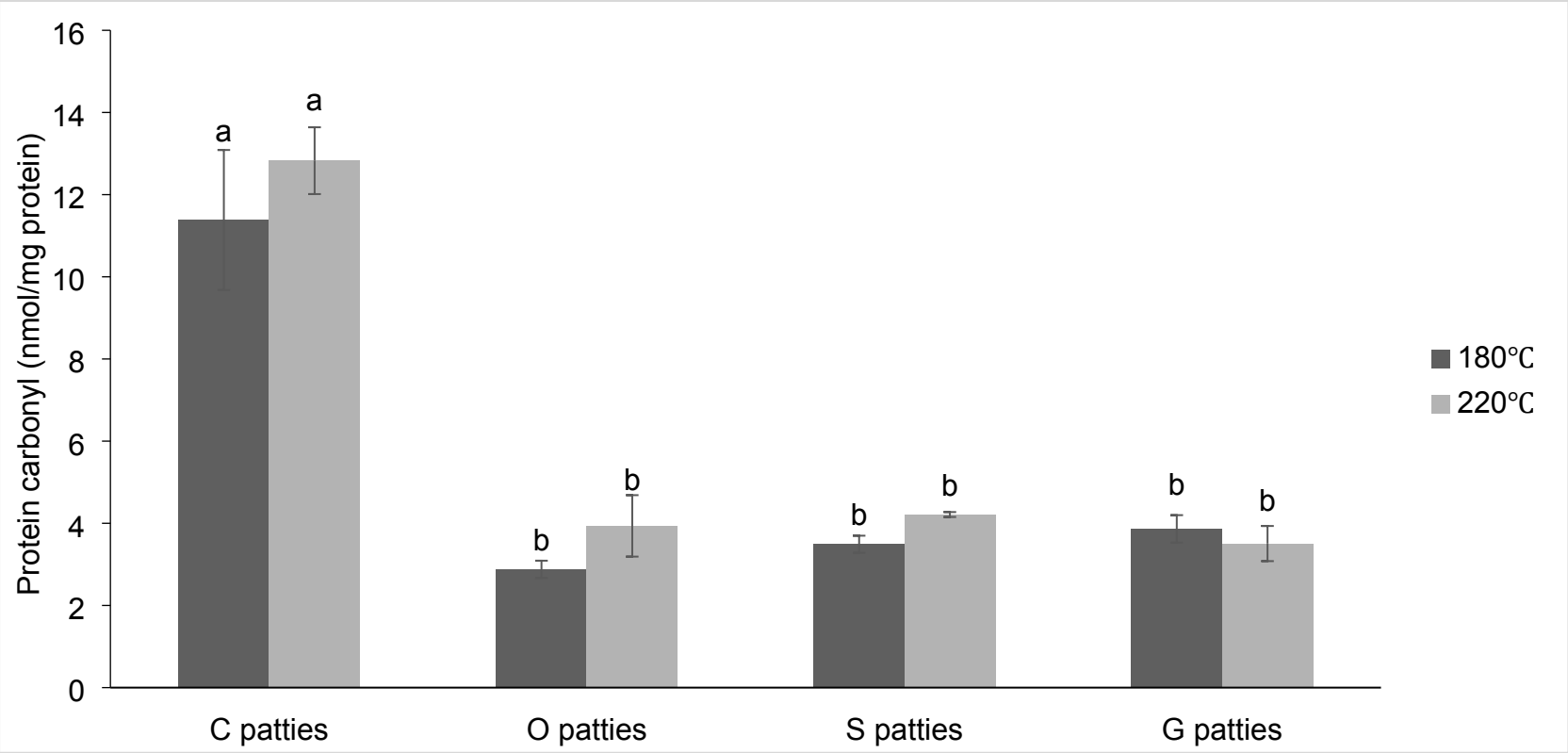
Figure 3: Protein carbonyl values in pork patties cooked at 180 ºC and 220 ºC.



Results with different letters are significantly different at the level p<0.05

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- Antioxidants in these oils could inhibit the formation of HCAs
- Grape seed oil achieved the highest inhibition capacity compared with sunflower oil and olive oil
- Lipid oxidation plays key role in the formation of HCAs
- Cooking temperature did not affect the total PAHs in pork patties

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