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

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1 **Vegetable oil as fat replacer inhibits formation of heterocyclic amines and**

2 **polycyclic aromatic hydrocarbons in reduced fat pork patties**

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7 **Abstract**

8 Formation of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons

9 (PAHs) was examined to evaluate the impact of using vegetable oil as fat

10 replacement on carcinogen formation in meat product. Pork patties were formulated

11 with 40% fat replacement by olive oil, sunflower oil or grape seed oil, respectively

12 and cooked at 180°C or 220°C. Control patties contained the highest amount of

13 HCAs compared with all other patties at both temperatures. Olive oil and sunflower

14 oil replacement completely inhibited formation of MeIQ (2-amino-3, 4-

15 methylimidazo[4,5-f]quinoline), while grape seed oil completely inhibited MeIQx (2-

16 amino-3,8-dimethylimidazo[4,5-f]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-

17 trimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-

18 b]pyridine) in patties. Grape seed oil achieved the highest inhibition capacity

19 compared with sunflower oil and olive oil. HCAs increased significantly with cooking

20 temperature ($p < 0.05$), but no difference was observed in total PAHs for patties

21 cooked at different temperature ($p > 0.05$). In conclusion, fat replacement with

22 sunflower oil, olive oil or grape seed oil in pork patties could reduce the formation of

23 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

2

48   Javidipour (2002) successfully substituted beef fat in Frankfurters with the mixture of

49   interesterified palm, cottonseed, and olive oil without changing physical parameters

50   and total sensory scores. Choi et al. (2010) used pre-emulsified grape seed oil and 2%

51   rice bran fibre to develop pork batters with 50% fat replacement and reported that

52   the fat-reduced pork batters could achieve the comparable eating quality with control

53   samples. Domínguez, Agregán, Gonçalves, and Lorenzo (2016) replaced 100% pork

54   back fat with olive oil in pork pâté, which significantly increase the content of

55   tocopherol and MUFA in cooked products without altering physio-chemical properties.

56   Domínguez, Pateiro, Agregán, and Lorenzo (2017) and Lorenzo, Munekata, Pateiro,

57   Campagnol, and Domínguez (2016) replaced 25%-75% backfat with olive oil,

58   microencapsulated fish oil and the mixture of fish oil and olive oil, which significantly

59   increased the percentage of PUFA in frankfurter type sausage and Spanish

60   *salchichón*. These results indicate that vegetable oil could be used successfully to

61   replace fat partially or completely to offer products comparable eating quality with

62   healthier fatty acids profile, i.e. high level of MUFA and PUFA.

63   However, unsaturated fatty acids in vegetable oils may pose risk in domestic cooking

64   due to their oxidation and decomposition at high temperature. For example, linoleic

65   acid was found associated with the formation of potentially toxic compounds, such as

66   free radicals, aldehydes and ketones (Guillén & Uriarte, 2012a; Katragadda et al.,

67   2010). These reactive oxygen species (ROS) initiated by unsaturated fatty acids

68   peroxidation could induce the decomposition of Amadori compounds and generate

69   1- and 3- deoxysone that are intermediates for Strecker aldehydes, pyrazines and

70   pyridines in Maillard reaction. Consequently, it might promote the formation of

71   heterocyclic amines (HCAs) (Morello, Shahidi & Ho, 2002; Turesky, 2010; Zamora &

72   Hidalgo, 2007). Effect of fatty acids/oils on the formation of HCAs has been

73   documented in previous research. Johansson et al. (1995) reported that the higher

74   level of MeIQx and DiMeIQx were found in burgers fried in rapeseed oil containing

75   high level of oleic acid with high peroxides values, compared with butter, margarine

76   and sunflower oil. Zamora et al. (2012) stated that both primary and secondary lipid

77   oxidation products, hydroperoxides, such as methyl 13-hydroperoxyoctadeca-9,11-

78   dienoate and alkenals could accelerate the formation of PhIP in chemical model

79   system. Some hydroperoxides generated from the decomposition of the unsaturated

80   hydrocarbons during heating, such as linolenate acid can also undertake

81   aromatization and de-hydrocyclization, further cleave into benzaldehydes and other

82   benzene ring-containing compounds, which are precursors of polycyclic aromatic

83   hydrocarbons (PAHs) (Chen & Chen, 2001; Lorenzo et al., 2011; Lorenzo, Purriños,

84   Fontán, & Franco, 2010; Singh, Varshney & Agarwal, 2016).

85   HCAs, PAHs and N-nitrous compounds are well-known carcinogens which were

86   detected in processed meat products (Hasnol, Jinap & Sanny, 2014; Jinap et al.,

87   2013; Liao et al., 2010; Oz & Kaya, 2010; Salmon, Knize & Felton, 1997). HCAs are

88   mainly formed with the presence of free amino acids, carbohydrates and creatine

89   under high cooking temperature (Rahman et al., 2014). IARC (1993) classified the

90   following 5 aminoimidazoarenes (AIAs) compounds as human carcinogens, including

91   2-amino-3-methylimidazo[4,5-f]quinoline   (IQ),   2-amino-3,4-methylimidazo[4,5-

92   f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-

93   3,4,8-trimethylimidazo[4,5-f]quinoxaline   (4, 8-DiMeIQx)   and   2-amino-1-methyl-6-

94   phenylimidazo[4,5-b]pyridine (PhIP). PAHs are hydrocarbons that contain two or

95   more benzene rings, such as pyrene, anthracene and naphthalene. They can be

96   formed through incomplete combustion or pyrolysis of organic components, including

97   fat, protein and carbohydrates at the temperature over 200 °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.

123 Excess visible fat on pork legs was trimmed, then minced by a Kenwood Food

124 processor (Chef Titanium KM010, 4.6, Kenwood Limited) and vacuum packed

125 separately. Raw materials were stored at -18 °C and defrosted 24h at 4 °C before

126 use. Commercial grape seed oil (Waitrose©, produced in Italy) with 12.4% SFA, 20.2%

127 MUFA, 68.2% PUFA, 10-15mg tocopherols and 5.9-11.5mg/100g polyphenols (Bail,

128 Stuebiger, Krist, Unterweger, & Buchbauer, 2008), sunflower oil (Morrisons©,

129 produced in UK) with 14.3% SFA, 20.5% MUFA, 63.3% PUFA and 50mg

130 tocopherols (McCance & Widdowson, 2002) and refined olive oil (Filippo©, phenols

131 were removed by industrial process, produced in Italy) with 14.3% SFA, 73.0%

132 MUFA, 8.2% PUFA and 100-300mg tocopherols (McCance & Widdowson, 2002)

133 were purchased from local supermarket (Reading, UK). Oils were kept in refrigerator

134 (4°C) before making patties and further analysis.

135 The standards IQ (2-amino-3-methyl-imidazo [4,5-f] quinoline), MeIQ (2-amino-3,4-

136 dimethyl-imidazo [4,5-f] quinoline), MeIQx (2-amino-3,8-dimthylimidazo [4,5-

137 f]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-f] quinoxaline),

138 PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine, BaA (Benz[a]anthracene)

139 and BaP (benzo[a]pyrene) were purchased from *Toronto Research Chemicals*

140 (Toronto, Canada). Ammonium acetate, triethylamine, acetonitrile (HPLC grade),

141 bovine serum albumin (BSA), dinitrophenylhydrazine (DNPH), ethyl acetate 99.5%

142 0.9000g/ml, 6M guanidine HCl (pH 6.5), hydrochloric acid solution 0.1M, methanol

143 (HPLC grade), HPLC grade water, sodium hydroxide 1M, perchloric acid (99.8%),

144 sodium phosphate buffer (pH 6.5), thiobarbituric acid (TBA), and trichloroacetic acid

145 (TCA) were purchased from *Fisher Scientific* (Loughborough, UK). 2,2-Azobis(2-

146 methylpropionamidine) dihydrochloride granular 97% (ABAP), 2,2-Azino-bis(3-

147 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-Hydroxy-

6

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).

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.

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°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).

2.4 Lipid/protein oxidation and antioxidant capacity

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 °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.

194 **2.4.2 Total protein carbonyl value (Protein oxidation)**

195 The degree of protein oxidation can be evaluated by calculating the total carbonyl

196 value according to the method described by Rodríguez-Carpena et al. (2012). 1g of

197 well blended pork patties was homogenized at 1:10 (w/v) in 20 mM sodium

198 phosphate buffer containing 0.6 M NaCl (pH 6.5) for 30 s. Two equal aliquots of

199 0.2ml mixture were then dispensed in 2 ml eppendorf tubes, respectively. 1ml cold

200 trichloroacetic acid (TCA) (10%, w/w) was added into tubes and centrifuged for 5 min

201 at 5000 rpm. One pellet was mixed with 1 ml 2 M HCl in order to measure protein

202 concentration, while the other pellet was mixed with 1 ml of 0.2% (w/v)

203 dinitrophenylhydrazine (DNPH) in 2 M HCl in order to measure carbonyl

204 concentration. Both tubes were incubated for 1 h at room temperature. Subsequently,

205 1 ml 10% TCA was added into tubes and pellets were washed twice with 1 ml

206 ethanol: ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were then

207 mixed with 1 ml of 20 mM sodium phosphate buffer containing 6 M guanidine HCl

208 (pH 6.5), stirred and centrifuged for 2 min at 5000 rpm to remove insoluble fragments.

209 Protein concentration was calculated from absorption at 280 nm using BSA as

210 standard. The amount of carbonyls was expressed as nmol of carbonyl per mg of

211 protein using an absorption coefficient of 21.0 nM^{-1} cm^{-1} for absorbance at 370 nm

212 for DNPH.

213 **2.4.3 *Trolox* equivalent antioxidant capacity (TEAC) of vegetable oil/ back fat**

214 TEAC was used to evaluate the total antioxidant capacity of vegetable oils and pork

215 back fat. The measuring procedures were based on the method reported by van den

216 Berg et al. (1999). An ABTS radical solution was prepared by mixing 2.5 mM ABAP

217 with 20 mM $ABTS^{2-}$ stock solution in 100 mM phosphate buffer (pH 7.4), which

218 contained 150 mM NaCl. The solution was covered with foil and heated at 60°C for

219 12 min, then cooled down to room temperature. 40 µl of the sample solution was

220 mixed with 1960 µl of the freshly prepared ABTS/ABAP solution. Difference of

221 absorbance at 734 nm in 6 min was recorded. A calibration curve was made by

222 measuring the difference of absorbance in 6min for *Trolox* at the concentration of 0,

223 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 µM. The TEAC of vegetable oil or back fat was

224 presented on a molar basis to *Trolox* (µmol Trolox/100g).

225 **2.5 Analysis of HCAs**

226 HCAs extraction and purification were following the procedures reported by

227 Puangsombat et al. (2011). To minimize the variation and bias due to the unevenly

228 distribution of sauce on the surface of meat, all samples were blended well with

229 sauce before measuring. 3g ground meat sample was mixed with 12ml 1M sodium

230 hydroxide firstly, then the mixture was transferred into an *Extrelut 20 column* with

231 17g diatomaceous earth. The HCAs were eluted by 60ml ethyl acetate in *Extrelut*

232 *column*, and transferred into PRS cartridge which was pre-conditioned with 7ml ethyl

233 acetate. A PRS cartridge was then washed with 6ml 0.1M HCl, 15ml methanol/0.1M

234 HCl (45/55, v/v) and 2ml pure water to remove interferences from the PRS cartridge.

235 The HCAs were then eluted by 20ml 0.5M ammonium acetate (pH 8.5) from the PRS

236 cartridge and transferred into a C-18 cartridge that was conditioned with 5ml

237 methanol and 5ml pure water. Finally, HCAs were eluted with 1ml

238 methanol/ammonium hydroxide (9/1, v/v) from C-18 cartridge into 2ml vial, followed

239 by drying the mixture under nitrogen stream for 1.5h at room temperature. The

240 residue in the vial was dissolved with 1ml methanol and submitted for HPLC analysis.

241 1ml of mixtures containing 5 standard compounds (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx

242 and PhIP) (5 ng/ml) was spiked into samples before extraction for measuring the

243 recovery rate. Three replicates were carried out for each sample.

244 IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP were analysed using HPLC (HP1635

245 Series, Agilent ChemStation, Agilent Technologies, Kidlington, UK) connected with a

246 diode array UV detector (RF 2000). The HCAs were separated gradually by a

247 reversed-phase Luna 5u C18 column (250 × 4.60 mm, 5 μm, 100A, Product No:

248 00G-4252-E0, Phenomenex, UK). Mobile phase A was 0.01 M triethylamine

249 (adjusted pH 3.6 with phosphoric acid) and phase B was acetonitrile (>99%, HPLC

250 grade). The solvent contained 95% A and 5% B at beginning, then linearly changed

251 to 75% A and 25% B within 30 min at flow rate 1.0 ml/min. The temperature of

252 column was 40°C. The UV detector was set at 252 nm (Puangsombat et al., 2011).

253 **2.6 Analysis of PAHs**

254 5g meat sample was homogenised with 15ml 1M NaOH for 1 h. The homogenized

255 sample was then mixed with 17g diatomaceous earth and loaded in an *Extrelut 20*

256 *column*. Elution of PAHs started from *Extrelut column*, and was followed by propyl

257 sulphonic acid (PRS) cartridges with 60 ml of CH_2Cl_2 containing 5% toluene. The

258 dichloromethane solution was then evaporated to small amount (0.5-1ml) and the

259 rest of the solvent was dried under a nitrogen stream. The residue was re-dissolved

260 in 1ml n-hexane and transferred to the top of a glass column packed with silica-gel

261 (10 g). PAHs were then eluted by 25 ml of n-hexane and 60 ml of 60:40 (v/v) n-

262 hexane–CH_2Cl_2 mixtures. After evaporation to dryness the residues were dissolved

263 in acetonitrile (spiked and unspiked samples) before the HPLC analysis (Janoszka,

264 2011). 50ng of 2 standard mixtures (BaA and BaP) was spiked for measuring the

265 recovery rate.

266   BaA and BaP were analysed using HPLC (HP1635 Series, Agilent ChemStation,

267   Agilent Technologies, Kidlington, UK) connected with a fluorescence detector.

268   Mixture of 84% acetonitrile (>99%, HPLC grade) and 16% water (HPLC grade) were

269   used as a mobile phase under isocratic conditions. The separations were performed

270   at 40°C under isocratic conditions with flow rate 1.0 ml/min. The fluorescence

271   detection was performed by applying the following excitation (Ex)/emission (Em)

272   wavelength program: 280/410 nm from 0 to 8.50 min (BaA), 376/410 nm from 8.50 to

273   15 min (BaP) (Janoszka, 2011).

274   **2.7 Recovery rate of HCAs and PAHs**

275   The 5 standard HCA (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) and 2 PAHs (BaA

276   and BaP) compounds were identified through the retention time of the peaks, and

277   the quantity of each individual compound was determined according to the standard

278   calibration curves, which was established by the standard solution at 0.5ng/ml,

279   5ng/ml and 50ng/ml. Limit of detection (LOD) for IQ, MeIQ, MeIQx, 4, 8-DiMeIQx,

280   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,

281   0.07 ng/g and 0.06 ng/g. The average recoveries of these 5 HCAs based on

282   triplicates were 60.01% for IQ, 61.76% for MeIQ, 53.64% for MeIQx, 60.57% for 4,8-

283   DiMeIQx and 55.98% for PhIP. Results were comparable with several published data

284   (Gibis, Kruwinnus & Weiss, 2015; Messner & Murkovic, 2004; Oz & Cakmak, 2016;

285   Yao et al., 2013). The recovery rate for BaA and BaP was 54.37 and 49.54%

286   respectively, which was comparable with published results of 50% - 115%

287   (Farhadian, Jinap, Abas, & Sakar, 2010; Ishizaki, Saito, Hanioka, Narimatsu, &

288   Kataoka, 2010; Iwasaki et al., 2010; Janoszka, 2011). Recovery rate could be

289   affected by sorbing materials, flow rate through cartridges, organic modifier quality

290   and/or content, interfering effects of eluting solvents (Busetti, Heitz, Cuomo, Badoer,

12

291 & Traverso, 2006). Stevens, Hamstra, Hegeman & Scharrer (2006) reported that

292 overlapping peaks on chromatogram caused by insufficient separation procedures

293 could result in recovery rate over 110%, and AOAC (2016) recommends that 40-120%

294 recovery rate is acceptable for compounds at 1ng/g concentration.

295 **2.8 Inhibitory rate of HCAs and PAHs**

296 Inhibitory rate was determined according to the equation:

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

298 where A_c was the total amount of HCAs/PAHs in control samples (ng/g), and A_t was

299 the total amount of HCAs/PAHs in fat partially replaced patties (S/ O/ G patties)

300 (ng/g).

301 **2.9 Physical parameters**

302 **2.9.1 Colour**

303 Colour feature including L*, a* and b* was measured using Hunter Lab Colour

304 instructment (Hunter Associates Laboratory, Virginia, USA, 2003). Each sample was

305 measured at 3 different locations, while the average was recorded as the colour

306 feature of the sample.

307 **2.9.2 Texture**

308 Texture profile analysis (TPA) was performed at room temperature with a *Stable*

309 *system* Texture Analyzer (Middleboro, USA).Cyclinder samples (D=18mm, H=22mm)

310 were prepared using a stainless cork borer. Before the analysis, samples were

311 tempered at room temperature (20°C) for 30min. The settings for texture analysis

312 were: load cell 5 kg, head speed 1.6 mm/s, and compression depth 10.0 mm. The

13

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**

14

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 °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 °C led to low moisture level in cooked patties, compared with low cooking temperature at 180 °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

15

361 patties at 170 ºC for 18 min had cooking loss at 20.69%- 22.20%. Fat and protein

362 content ranged from 9.49%-10.01% and 15.03%-15.34% respectively, as expected,

363 fat and protein content in fat modified patties were comparable with these in C

364 patties. pH ranged from 5.85 to 5.99. Cooking temperature and type of oil did not

365 affect pH of patties, there was no interaction between temperature and type of oil as

366 well (p>0.05).

367 **3.1.2 Physical parameters**

368 **3.1.2.1 Texture**

369 Texture property of cooked pork patties was examined including hardness,

370 cohesiveness, springiness and chewiness through a typical texture profile analysis.

371 Results were listed in Table 3. Cooking temperature affected hardness,

372 cohesiveness and chewiness ($p \leq 0.05$), but had no effect on springiness (p>0.05).

373 Fat replacement with vegetable oils did not affect any of the texture attributes

374 (p>0.05). There was no interaction observed between oil replacement and

375 temperature for hardness, chewiness and springiness except cohesiveness (p>0.05).

376 High cooking temperature led to high hardness. Control patties cooked at 220°C had

377 significantly higher hardness (26.65±3.15N) than that cooked at 180°C (20.14±2.81N)

378 (p<0.05). Roldán et al. (2013) observed that the elevating cooking temperature

379 resulted in higher hardness, while the increased hardness in pork patties might be

380 associated with high cooking loss. There was no difference observed in hardness for

381 fat modified patties regardless of temperature (p>0.05), which agreed with the results

382 reported by Rodr´ıguez-Carpena et al. (2011). They stated that patties that partially

383 (50%) replaced with sunflower oil, olive oil and avocado oil had same hardness with

384 control patties. However, Hur, Jin and Kim (2008) reported that olive oil replacement

16

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 °C in water bath, while samples were cooked in convection oven at

388   180°C or 220°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°C had

400   higher cohesiveness than these cooked at 180°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 Rodŕıguez-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

17

410 processing (Ganhão, Morcuende & Estévez, 2010). Thus, grape seed oil/meat

411 emulsion was less sensitive to temperature changes in relation to cohesiveness of

412 the final products.

413 For chewiness, temperature significantly affected it (p<0.05). High cooking

414 temperature resulted in high chewiness. Chewiness remained similar for all patties

415 cooked at 180 ^{o}C (from 3.94 to 4.11N.s), but increased to 5.92±0.77 N.s in C patties,

416 and 5.35±0.41 N.s in S patties that cooked at 220 ^{o}C. Greater hardness,

417 cohesiveness and chewiness at higher elevating cooking temprature could be due to

418 the more severe denaturation of myosin (40-60 ^{o}C) and actin (66-73 ^{o}C) (Sánchez del

419 Pulgar et al., 2012). In addtion, chewiness is also associated with the water retention

420 in meat products (Roldán et al., 2014). Patties cooked at 180°C had significantly

421 higher mositure content than thoes cooked at 220°C (as shown in Table 2).

422 Therefore, high chewiness would be expected in samples cooked at 220°C due to

423 high moisture loss.

424 **3.1.2.2 Colour**

425 Effects of vegetable oil and cooking temperature on colour characteristics of cooked

426 pork patties including lightness (L*), redness (a*) and yellowness (b*) were

427 summarized in Table 3. Temperature significantly affected all three parameters (p ≤

428 0.05), especially a* with p=0.005. Different vegetable oils did not have any impact

429 on the colour parameters, while the interaction between oil and temperature was

430 observed in yellowness (b*). Patties cooked at 220 ^{o}C had lower L* than those

431 cooked at 180 ^{o}C (p<0.01), which agreed with the results of Sánchez del Pulgar et al.

432 (2012). The decrease in surface lightness could be attributed to the brown pigments

433 formed from caramelization of sugars and Maillard reaction when samples were

18

434 cooked at temperature over 90 °C (Girard, 1992). In addition, the lightness was also

435 associated with the moisture content in meat products. Qiao et al. (2001) reported

436 that there was positive correlation between lightness and moisture content in broiler

437 breast fillet. Presence of heme pigments, containing 90-95% myoglobin in muscles

438 gives meat red colour (a*). At 180 °C, a higher a* value was found in all pork patties

439 with oil replacement than control patties (p<0.05). The antioxidants in these

440 vegetable oils, such as vitamin E could prohibit the oxidation of oxymyoglobin and

441 lead to a high redness in the final products (Hui, 2001; Sánchez del Pulgar et al.,

442 2012). Cooking temperature could significantly affect a* as well. All patties that

443 cooked at 220 °C had significantly lower a* than those cooked at 180 °C. The

444 reduction of a* caused by increased temperature could be associated with the

445 denaturation of myoglobin (Nollet, 2012). Liao, Xu and Zhou (2009) found that a* of

446 stir fried pork floss decreased significantly by 30% when cooking temperature

447 increased from 125 °C to 150 °C.

448 Yellowness b* ranged from 15.41-18.73 in all cooked patties. Both type of oil and

449 cooking temperature had no effect on b* values, but the interaction between type of

450 oil and cooking temperature was observed. Jamali et al. (2016) also found that b*

451 value in beef patties was not affected by cooking temperature (160 °C and 220 °C).

452 The results of b* in control samples (16.98-18.73) were comparable with Vittadini et

453 al. (2005).

454 **3.2 Effects of vegetable oils and cooking temperature on the formation of**

455 **HCAs**

456 Concentration of HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) in control patties

457 and fat modified patties cooked at 180°C and 220°C were listed in Table 4. Type of

19

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 $\pm$ 0.98ng/g) and 4, 8-DiMeIQx

485 (43.37 $\pm$ 15.67ng/g) in C patties, while PhIP in S (14.78 $\pm$ 1.49ng/g) and O patties

486 (22.70 $\pm$ 1.95ng/g). The total HCAs in C patties were higher than some published

487 results. Vangnai et al. (2014) reported MeIQx (7.59 $\pm$ 0.43ng/g), PhIP (13.12 $\pm$ 0.72

488 ng/g) and total HCAs (22.35 $\pm$ 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

22

533 temperatures, there was no MeIQx detected. However, sunflower oil and olive oil did

534 not affect MeIQx in patties, although both oils were rich of vitamin E. No MeIQx was

535 detected at all which was in agreement with Rounds et al. (2012), who also reported

536 grape seed extract could completely inhibit the formation of MeIQx in cooked beef

537 patties. Temperature significantly increased formation of 4, 8-DiMeIQx as evidenced

538 in C patties cooked at 180 $^\circ$C and 220 $^\circ$C (25.66±1.51 ng/g and 43.37±15.67 ng/g)

539 (p<0.05). All vegetable oils effectively reduced 4, 8-DiMeIQx in patties. Grape seed

540 oil was the most effective one among the three vegetable oil as 4, 8-DiMeIQx was

541 not detected in G patties cooked at both temperatures.

542 PhIP ranged from Nd to 24.07±1.99 ng/g. A similar level of PhIP (18.4 $\pm$ 11.5 ng/g) in

543 the fried pork patties was reported by Zhang et al. (2013), when patties were cooked

544 at 180°C for 5 min. At 180 $^\circ$C, PhIP (11.43±6.33 ng/g) was totally inhibited by all 3

545 vegetable oils, but only grape seed oil could completely inhibit the formation of PhIP

546 at both cooking temperatures. The stronger inhibitory effect on PhIP in G patties

547 could be attributed to the phenolic compounds in grape seed oil. Gibis and Weiss

548 (2012), Jamali et al. (2016) and Oguri et al. (1998) found that catechin, epicatechin

549 and epicatechin-3-O-gallate in grape seed extract may be responsible for 50%-90%

550 reduction of PhIP in both oven cooked beef patties and chemical model system.

551 Zamora and Hidalg (2015) suggested phenolic compounds could effectively

552 scavenge the carbonyl compounds in the Strecker degradation of phenylalanine to

553 produce phenylacetaldehyde (major intermediate in the development of PhIP).

554 Temperature significantly affected PhIP level in pork patties (p<0.05). PhIP

555 increased significantly in C patties from 11.43±6.33 ng/g to 24.07±1.99 ng/g, O

556 patties from Nd to 14.78±1.49 ng/g and S patties from Nd to 22.70±1.95 ng/g when

557 cooking temperature increased from 180 $^\circ$C to 220 $^\circ$C (p<0.05). The results agreed

23

558 with Gibis and Weiss (2012) and Wong et al. (2012) that PhIP level was directly

559 related to the cooking temperature. At 175-200 °C, only very low level of PhIP (0-

560 6.91 ng/g) could be formed even at varied cooking time, but it could increase

561 dramatically to 31.80ng/g with prolonged cooking time if temperature went above

562 200 °C.

563 Type of vegetable oil significantly affected the level of total HCAs in cooked patties

564 (p<0.05), but temperature did not have any effect (p>0.05). Interaction between

565 types of vegetable oil and cooking temperature was observed as well (p<0.05). In the

566 current study, reduction of total HCAs by 85.75%-93.90% was found in O patties,

567 83.01%-91.15% in S patties, while G patties achieved the highest reduction rate at

568 98.64%-100% (Table 4). Antioxidants in the vegetable oils could be responsible for

569 the reduction of total HCAs as a strong negative correlation (r=-0.618, p<0.01) was

570 disclosed between total HCAs level and antioxidant capacity (TEAC) of oils (Table 6).

571 Grape seed oil had the highest TEAC value with 0.71±0.01 µmol Trolox/100g,

572 followed by olive oil (0.52±0.05 µmol Trolox/100g) and sunflower oil (0.18±0.04 µmol

573 Trolox/100g), while pork back fat had the lowest TEAC value (0.09±0.02 µmol

574 Trolox/100g) (Figure 1). Therefore, reduction of HCAs in vegetable oils, especially

575 grape seeds oil was expected compared with control sample. Results were

576 comparable with findings of Matthäus (2008) and Castelo-Branco and Torres (2012).

577 Balogh et al. (2000) found that 1% vitamin E spray on the surface of beef patties

578 could reduce the concentrations of IQ, MeIQ, MeIQx, DiMeIQx and PhIP significantly

579 by 45% to 75%, because vitamin E could remove free radicals in Maillard reactions.

580 Similar result was also reported by Lan, Kao and Che (2004). They found that 70%

581 of total HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) were prohibited when 0.2%

582 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°C had significantly higher level of toal HCAs than these at

593 180°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 °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°C). Olive oil and grape seed

613 oil showed inhibitory effect on BaP when patties cooked at 220 °C, but no effect or

614 even promoting effect was observed at 180 °C. On the contrast, sunflower oil offered

615 inhibition on BaP at 180 °C, but promotion at 220 °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 °C and S patties cooked at 180 °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 °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 °C and C

626 patties (3.28 $\pm$ 0.07 ng/g) cooked at 220 °C. O patties cooked at 220°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°C, sunflower oil 227°C) which makes them easy to

26

632 decompose, compared with olive oil (smoke point 242°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.

3.4 Correlation between lipid oxidation, protein oxidation and the formation of

HCAs and PAHs

656   In Figure 2, S patties, O patties and G patties had significantly lower TBARS values

657   than control samples (p<0.05), while G patties had the lowest TBARS value

658   compared with O and S patties (p=0.001). This inhibitory effect against lipid oxidation

659   could be attributed to the antioxidants (tocopherols and phenolic compounds) within

660   the oils, since a significantly negative relationship was found between TBARS values

661   and antioxidant capacity of oils (r= -0.764, p<0.01). Wong et al. (2015) reported that

662   0.1-0.4mmol vitamin E could inhibit 45% of lipid oxidation in beef patties, by

663   obstructing the formation of some key aldehydes and ketones during lipid oxidation.

664   Similar results were achieved by Ahn, Grün and Fernando (2002) as well. Frankel

665   (1998) proposed that α-tocopherol could prevent the chain propagating and remove

666   free radicals through reacting with either singlet oxygen or peroxyl radicals.

667   Consequently lipid oxidation was reduced. Meanwhile, polyphenols, such as

668   epicatechin (EC) oligomer procyanidins were also sufficient to inhibit lipid oxidation

669   by reducing free radicles and preventing chain propagation in cooked pork and beef

670   (Rojas & brewer, 2007). They could also chelate metals (iron and cooper in meat) or

671   react with ROS, and then turn into non-radical species. As a result, reactions were

672   terminated (Roman et al., 2013). Moreover, Ahn et al. (2002) and Tang et al. (2001)

673   reported that polyphenols such as catechin, epicatechin were more efficient in

674   inhibiting lipid oxidation than α-tocopherol at the same concentration in cooked meat.

675   This could explain why G patties had the lowest TBARS values than S and O patties.

676   Although Gunstone (2002) stated that a higher degree of unsaturation of fatty acids

677   could be easier to trigger the lipid oxidation and interacted with Maillard reaction, the

678   presence of antioxidants should be also considered in the formation of HCAs.

679   Protein carbonyls are produced from protein oxidative degradation in meat products,

680   which were used to analyse degree of protein oxidation (Figure 3). Significant effect

28

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

730 observed between TBARS /protein carbonyl and PAHs (p>0.05), which indicated that

731 the involvement of lipid and protein oxidation in the formation of PAHs was only at

732 null level. Thus, no linear regression model was fitted. The antioxidants in vegetable

733 oils could not inhibit the formation of PAHs, which was evidenced by null correlation

734 between TEAC and PAHs (p>0.05).

735 **4. Conclusions**

736 Control patties contained the highest amount of HCAs and relatively higher PAHs at

737 180 ^{o}C and 220 ^{o}C. All 3 fat modified patties had significantly lower HCAs, which

738 could be attributed to antioxidants, such as tocopherols and polyphenol compounds

739 existing in the oils. The negative correlation (r= -0.618, p<0.01) between the

740 antioxidant capacity of lipids and the total amount of HCAs could be useful evidence

741 to support this claim. Both lipid and protein oxidation contributed to the formation of

742 HCAs, which were supported by the positive relationship between TBARS/ protein

743 carbonyl values and total HCAs with r= 0.826 and 0.788 (p<0.01), respectively. Olive

744 oil and sunflower oil completely prohibited MeIQ, whereas grape seed oil could

745 inhibit MeIQx, 4, 8-DiMeIQx and PhIP. Grape seed oil could achieve the highest

746 inhibitive effect on the formation of HCAs. However, effect of vegetable oils on the

747 formation of PAHs was not consistent, which could be attributed to complexity of oil

748 decomposition and antioxidants in the oils. The involvement of lipid oxidation and

749 protein oxidation in formation of PAHs was limited or at a minimum level.

750 Antioxidants in oils could not reduce the total amount of PAHs effectively. Therefore,

751 it is necessary to explore other methods to reduce PAHs in processed meat. Due to

752 the synergistic effect of antioxidants, it would be interesting to explore replacing pork

753 back fat with a mixture of several types of oils to reach an optimum fatty acids profile,

31

754 instead of replacing pork back fat with one single type of oil. Overall, replacing pork

755 back fat with vegetable oils in processed meat products could offer healthier meat

756 products with reduced HCAs without compromising eating quality.

757 **Acknowledgement**

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1127 Table 1: Formulation of control and fat reduced pork patties

Treatment	Ingredients in recipe represent as g/1kg				
	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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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

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

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1138 Table 3: Texture parameters (hardness, cohesiveness and chewiness) and colour parameters (lightness L*, redness a* and
1139 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

1140 [a] Results with different letters in the same column are significantly different at the level $p<0.05$.
1141 [b] Values represented as the Mean ± SD, n=3.

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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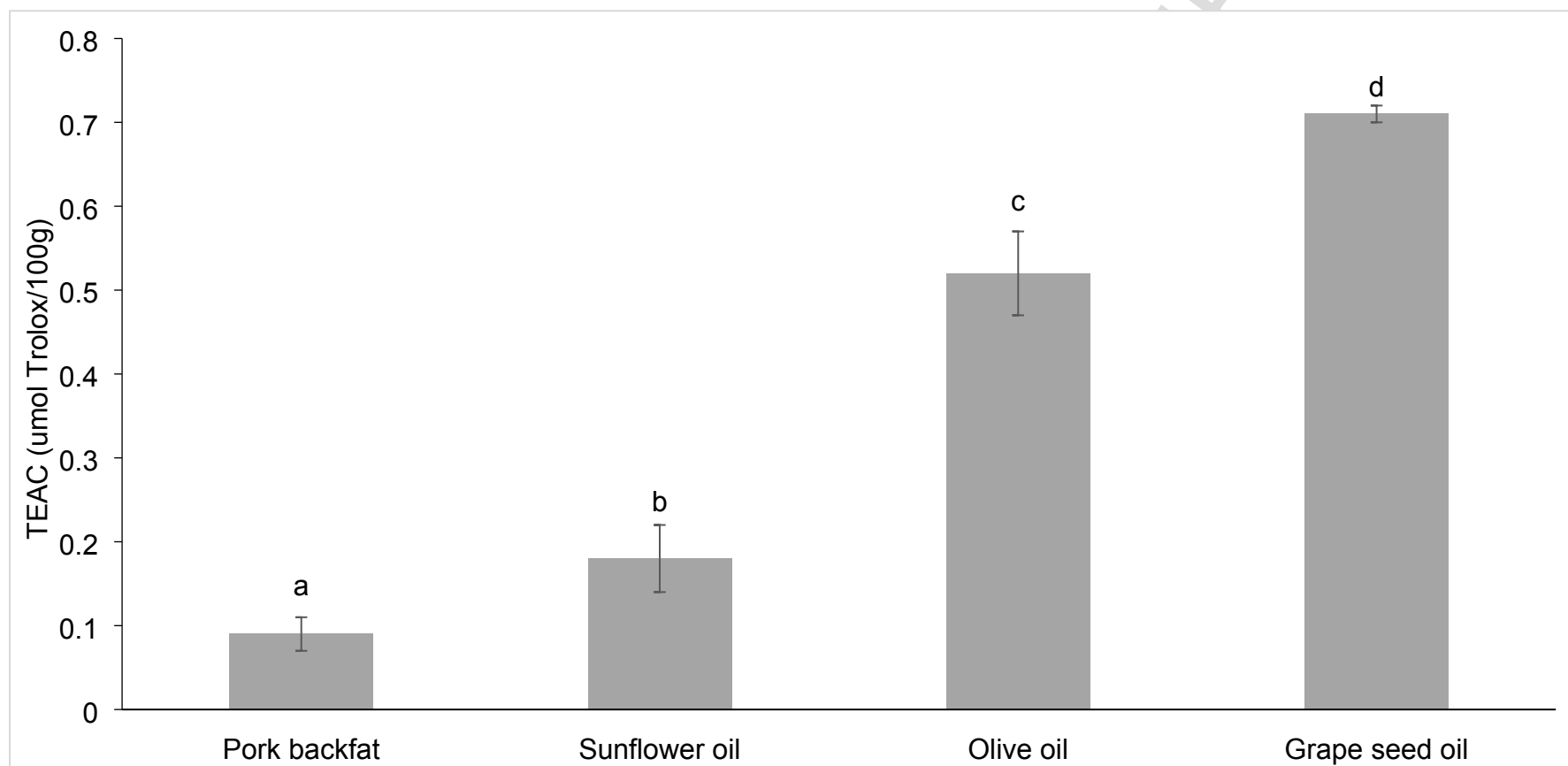1152 Table 6: Pearson correlation coefficient (p) between the level of total HCAs/PAHs (ng/g) and TBARS, protein carbonyl and TEAC

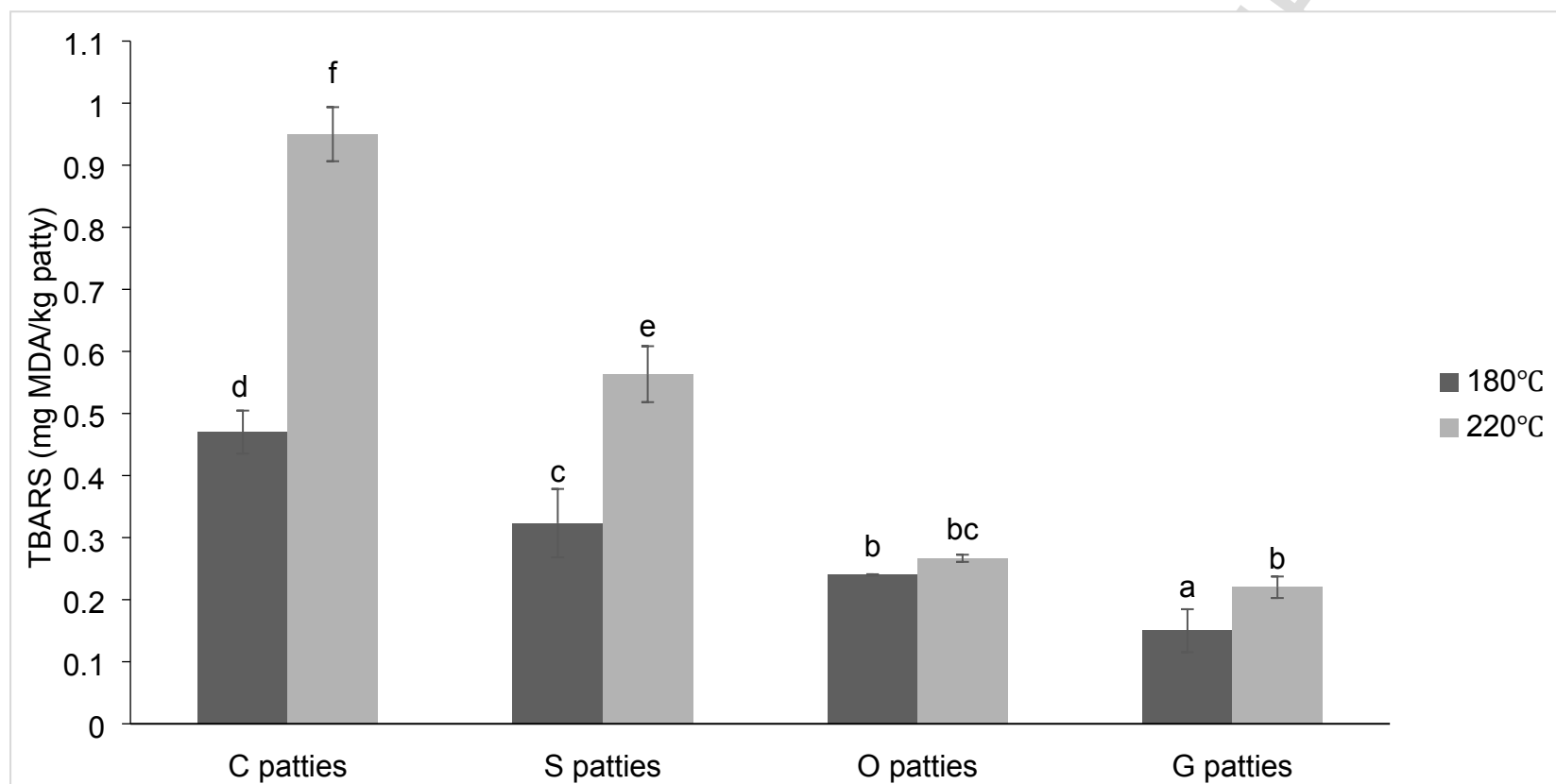|	TEAC	HCAs	PAHS
TBARS	-0.764**	0.826**	-0.154
Protein carbonyl	-0.606**	0.778**	0.019
TEAC	-	-0.618**	0.301

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.
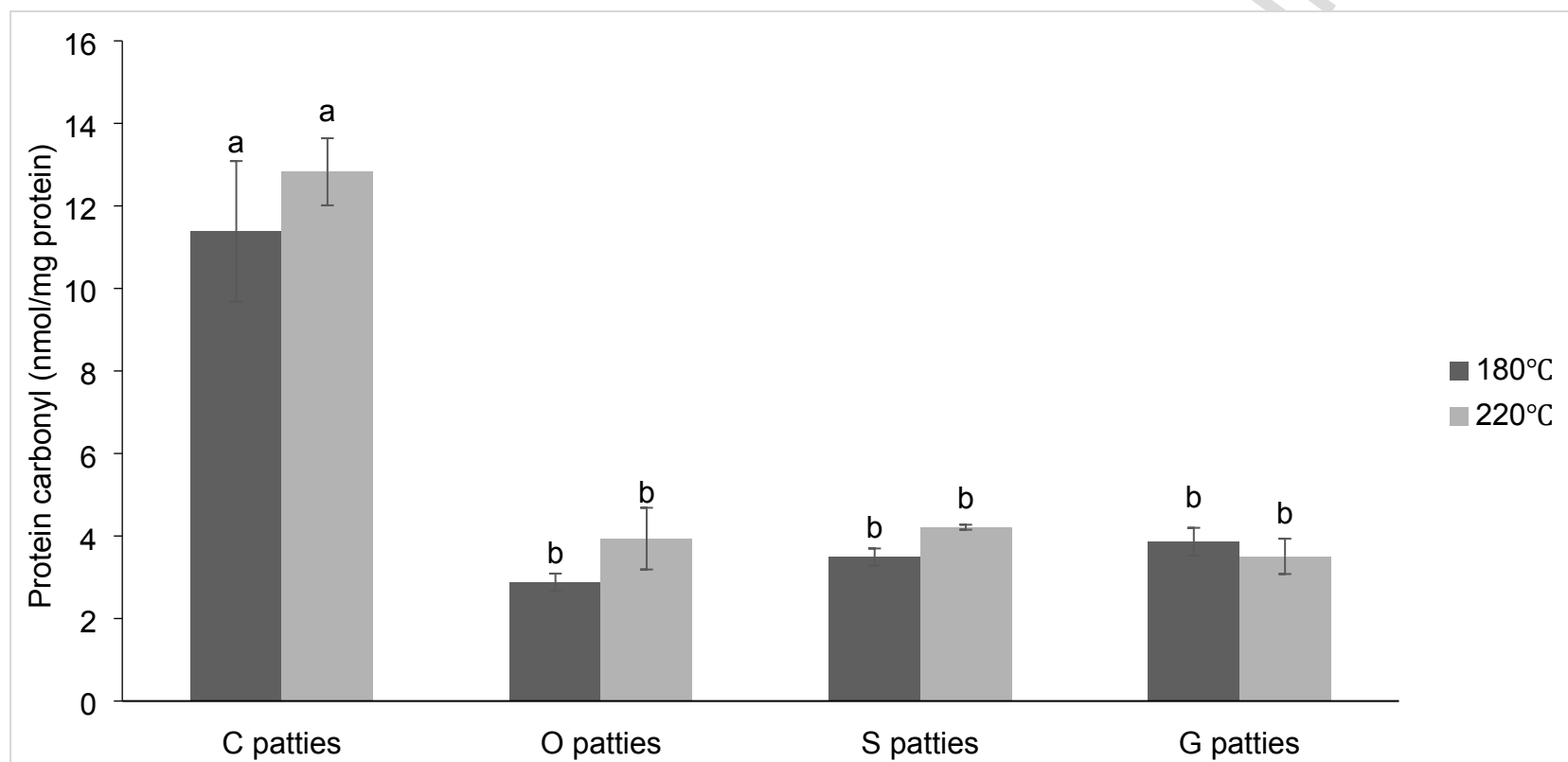

Results with different letters are significantly different at the level p<0.05.

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

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

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

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

- 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