

1 **Beetroot improves oxidative stability and functional properties of processed foods: Singular**
2 **and combined effects with chocolate.**

3
4
5 **Research highlights**

- 6
- 7 • Beetroot improved nutrition, oxidative stability and shelf-life of sponge cake
 - 8
 - 9 • Beetroot's effects were enhanced when combined with chocolate.
 - 10
 - 11 • Chocolate reduced lipid oxidation during gastrointestinal digestion.
 - 12
 - 13 • Beetroot and chocolate addition did not affect cake texture, and delayed staling.
 - 14
 - 15
 - 16
 - 17
 - 18
 - 19
 - 20
 - 21
 - 22
 - 23
 - 24
 - 25
 - 26
 - 27
 - 28
 - 29
 - 30
 - 31
 - 32
 - 33
 - 34
 - 35
 - 36
 - 37
 - 38
 - 39
 - 40
 - 41
 - 42
 - 43
 - 44

45 **ABSTRACT:**

46 Oxidation is a significant problem in processed foods affecting their physico-chemical, shelf life
47 and health properties. Natural antioxidants could be viable alternatives to synthetic variants for
48 safely improving antioxidation properties of processed foods. The aim of this study was to assess
49 the singular and combined effects of beetroot and chocolate on the oxidative stability of a high
50 fat and protein processed food (sponge cake) during storage and gastrointestinal digestion. Cakes
51 were prepared and assessed for antioxidant potential, polyphenols, and oxidative stability, and
52 macronutrient oxidation during simulated gastro-intestinal digestion. Beetroot significantly
53 improved the antioxidant and polyphenol profiles of sponge cake which further improved with
54 chocolate addition. Beetroot also significantly increased the oxidative stability and shelf-life of
55 sponge cake, and these effects were enhanced when combined with chocolate. Chocolate
56 significantly reduced lipid oxidation during the gastric phase of digestion. However, both
57 chocolate and beetroot did not curtail lipid oxidation in the intestinal phase, nor protein
58 oxidation at any of the phases. Promisingly, beetroot and chocolate addition did not affect
59 textural parameters and delayed staling by up to two days. Overall, the benefits of beetroot and
60 chocolate addition were manifested more in the food system than during its digestion. Beetroot
61 improves the oxidative stability and shelf life of processed foods, and its effects could be
62 enhanced through combining with other natural products.

63

64 **Keywords:** Beetroot, chocolate, oxidation, antioxidants, oxidative stability, gastrointestinal
65 digestion, processed foods

66

67

68 **Introduction**

69 Processed foods remain popular and are widely consumed by all segments of the population. The
70 high protein and fat contents often seen in processed foods increase their susceptibility to
71 oxidation, affecting their physico-chemical characteristics and shelf life. End-products of
72 macronutrient oxidation have been shown to also adversely affect health. For instance,
73 aldehydes such as malondialdehyde resulting from the oxidation of fatty acids exert mutagenic
74 and atherogenic effects, while protein oxidation products such as carbonyls promote cell ageing
75 and age-related diseases (Miyata et al. 1998, Niedernhofer et al. 2003). Synthetic antioxidants
76 are often added to processed foods to curtail macronutrient oxidation, although they have been
77 implicated in exacerbating disease (Shahidi and Ambigaipalan 2015).

78

79 Food reformulation strategies for improving the health properties of processed foods are being
80 increasingly adopted in response to consumer and public health demands for healthier diets
81 (Leroy et al. 2015). Natural products in particular are being increasingly used in food
82 reformulations for the multiple benefits they confer both to consumers and manufacturers.
83 These include improved nutritional profiles and producing 'clean label' products. Our work has
84 looked at the potential of beetroot in this regard as it is rich in phytochemicals with demonstrated
85 nutritional and antioxidant properties (Clifford et al. 2015). Some of these effects were
86 confirmed in our work on bread, burgers and mayonnaise where beetroot addition had several
87 potentially beneficial product specific effects including decreasing fat and protein oxidation, and
88 improving anti-oxidant potential, oxidative stability and shelf-life (Duthie et al. 2013, Raikos et al.

89 2015, Ranawana et al. 2016a). However, its effects appear to be product specific, possibly
90 mediated by food composition and processing conditions.

91
92 Numerous studies have demonstrated the antioxidant properties of chocolate (Goya et al. 2016),
93 however there is limited data on how its addition into processed foods affect oxidative stability
94 and functional attributes. The objective of the present study was to further assess the effects of
95 adding beetroot on functional and chemical antioxidant properties of processed foods, and to
96 determine its combined effects with chocolate. Sponge cake was selected as the model as it is a
97 high fat and protein food that is widely consumed. Beetroot and chocolate are ingredients
98 conventionally used in cake, which further supports its suitability as a test model.

99
100 The specific aims of the study were to assess the singular and combined effects of beetroot and
101 chocolate on the oxidative stability properties of sponge cake and during its gastro-intestinal
102 digestion. The study hypothesised that the addition of beetroot or chocolate would improve the
103 functional antioxidant properties of sponge cake and show cumulative benefits when combined.

104
105 **Materials and Methods**

106 **Chemicals and reagents**

107 The reagents used were: Na₂PO₄, KH₂PO₄, NaCl, pancreatin, pepsin, mucin, α-amylase, Adenosin
108 diphosphate, Trichloroacetic acid, folin and ciocalteu's phenol reagent , Na₂CO₃, Gallic acid,
109 1,1,3,3-tetramethoxypropane (TMP), thiobarbituric acid, sodium hydroxide, glacial acetic acid,
110 300mM acetate buffer, HCl, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), FeCl₃, 0.2% 2,4-

111 dinitrophenylhydrazine (DNPH), 6M guanidine hydrochloride,. All reagents were of analytical
112 grade and sourced from Sigma-Aldrich Co Ltd. (Dorset, UK) unless otherwise stated.

113

114 Preparation of beetroot and cakes

115 Small to medium sized fresh beetroots (Globe var) were purchased from a local supermarket
116 (Sainsbury's Supermarkets Ltd, London, UK) and were washed and dried. The beetroots were
117 pricked with a fork, sprinkled with water, placed covered in a microwavable dish and cooked for
118 seven minutes at 60% power and three minutes at 80% power in a commercial microwave oven
119 (1600 Watts; CF359, Buffalo Appliances, Bristol, UK). The cooled beetroot was peeled, ground to
120 a puree and passed through a sieve to ensure no particles were present, and used immediately
121 in the preparation of the cakes. The cocoa used was a standardised brand (Cadbury Bourneville,
122 Cadbury, Birmingham, UK) and previously characterised for polyphenol content (Santos and Coe
123 2016).

124

125 The study evaluated four types of cakes; chocolate cake with beetroot (CB), chocolate cake
126 without beetroot (CN), plain cake with beetroot (PB) and plain cake (PN). The cake formulation
127 used is typical of what is used in commercial production (Campbell et al. 2016). For preparing the
128 two beetroot cakes (Table 1) the sugar, oil and egg were beaten for two minutes using a hand-
129 held beater until homogenous. The beetroot puree was folded in followed by the dry ingredients.
130 For the beetroot-free cakes, the sugar, oil and egg were beaten for two minutes using a hand-
131 held beater until homogenous. Then dry ingredients were folded in. Finally the water was added
132 and the batter mixed until smooth. The cake batters were poured into greased and lined loaf tins

133 (11 cm x 21cm) and baked in a non-fan-assisted oven at 180 °C for 45 minutes. The cooked cakes
134 were cooled in the tins for 5 minutes before unmoulding and cooling on a wire rack. For the
135 antioxidant, polyphenol and oxidative stability experiments samples of the cooled cakes were
136 immediately freeze dried (Model HS1, Frozen in Time Ltd., York, UK) and used. Fresh cake
137 samples were used for the digestions and for texture measurement.

138

139 Measuring antioxidant capacity and total polyphenol content

140

141 Preparation of sample extracts

142 1 gram of sample (ground freeze dried cake powders) was added to eppendorfs containing 10mL
143 of 0.9% NaCl. An aqueous isotonic extraction medium was selected as this was more
144 physiologically relevant. The suspensions were mixed on a roller for 30 minutes, sonicated in a
145 water bath for 30 minutes and centrifuged at 6000rpm (CR312, Jouan, Thermo Fisher Scientific,
146 Renfrew, UK) for 10 minutes. The procedure was repeated to ensure maximum extraction,
147 combined and stored at -70°C until analysed for antioxidant potential and total polyphenol
148 content.

149

150 Analysis of total antioxidant potential and polyphenol content

151 Antioxidant potential was measured using the Ferric ion Reducing Antioxidant Power (FRAP) and
152 total phenolics in the extracts was estimated according to methods described earlier (Raikos et
153 al. 2016).

154

155 Oxidative stability of cakes

156 The Rancimat method was used to measure susceptibility to lipid oxidation. The 743 Rancimat
157 model (Metrohm Ltd, Herisau, Switzerland) measures the resistance of food products containing
158 fats and oils to oxidative rancidity, and thus provides an indication of shelf life. Freeze dried cake
159 samples (2.5g) were transferred to the reaction vessels and subjected to an accelerated oxidation
160 at 120 °C and an ambient air flow rate of 20L/h.

161

162 Simulated Gastro-intestinal digestions

163 The method used has been described elsewhere (Ranawana et al. 2016a, Ranawana et al. 2016b).
164 Cake samples were weighed into 15 mL black centrifuge tubes (LightSafe, Sigma-Aldrich, Dorset,
165 UK) and 3 mL of cold simulated saliva was added. The digestion tubes were incubated at 37 °C
166 for 5 minutes to complete the oral phase of digestion (pH 6.8) and the phase halted by the
167 addition of 0.3M HCl. The gastric phase of digestion was subsequently initiated by the addition
168 of SGF (pH 2.0) which contained 0.68 mg of ascorbic acid, 0.11 mg of FeSO₄ and 6.8 mg of ADP to
169 create a pro-oxidant environment. The gastric digestion phase was continued for four hours,
170 aliquots being transferred at two hours to tubes containing SIF (pH 8.0) to simulate intestinal
171 digestion phase for two hours. At baseline, and during each of the digestion phases digesta were
172 transferred into, (1) glass tubes containing 20% trichloroacetic acid for measuring concentrations
173 of TBARS and, (2) glass tubes containing 0.2% dinitrophenylhydrazine (DNPH, in 3.5 M HCl) for
174 measuring protein carbonyls (PCs). Digesta samples collected for TBARS quantification were
175 analysed immediately following the digestions whilst those collected for PC analysis were stored

176 at -70 °C and analysed within 7 days. All of the cake samples were subjected to *in vitro* digestions
177 in three independent runs and the data pooled for analysis.

178

179 Measurement of thiobarbituric acid reactive substances (TBARS) in digesta samples
180 Concentrations of TBARS were analysed by the method described previously (Duthie et al. 2013)
181 using high performance liquid chromatography. One millilitre of freshly prepared thiobarbituric
182 acid reagent (0.67g thiobarbituric acid, 100 mL glacial Acetic acid in 200 mL solution) was added
183 into digesta sample tubes and the contents heated for 30 minutes at 90-100°C. Cooled samples
184 were centrifuged and the supernatants analysed using HPLC using a Waters 2695 Separations
185 Module (Waters Corporation, Milford, USA) equipped with a Waters 2475 fluorescence detector
186 (Waters Ltd, Elstree, UK) and a Luna® 5µm C18 (2) 100 Å, 100 x 4.6mm column (Phenomenex,
187 Cheshire, UK). TBARS were determined with isocratic elution at a flow rate of 0.6ml/min, sample
188 run was 15 minutes, injection volume was 20µl and fluorescence detector wavelengths were set
189 to 515nm (excitation) and 546nm (emission). The mobile phase consisted of 60% (v/v) KH₂PO₄
190 (50mM, pH 7.0) and 40% (v/v) methanol. Standard solutions of TMP was used for constructing
191 calibration curves and quantification of TBARS (concentration range: 0-2 mMol/L).

192

193 Measurement of protein carbonyls in digesta

194 Protein carbonyls in the digesta samples were analysed as previously described (Duthie et al.
195 2013). The digesta samples in 0.2% DNPH were heated at 45°C for 1 hour and centrifuged at
196 13,000xg for 5 minutes. The supernatant was removed and discarded and ethanol:ethyl acetate
197 (1:1 v/v) was added to re-dissolve the pellet. The samples were incubated at room temperature

198 for 10 minutes whilst vortexing occasionally. The centrifuging, supernatant removal and washing
199 procedure was repeated a further two times. The pellet was re-dissolved in 300µl 6M guanidine
200 hydrochloride and absorbance read at 370nm (µQuant, Bio-Tek instruments Inc, Winooski, USA),
201 and PC content quantified using the molar extinction coefficient of 22,000M⁻¹cm⁻¹.

202

203 Texture analysis of fresh cakes and during storage

204

205 Freshly baked and cooled cakes were cut into 20 mm thick slices and 3 randomly selected slices
206 from each type were placed in air tight plastic containers. One set of samples was analysed on
207 the day of baking for baseline measurements (day 0). The remainder were stored for 1, 2 and 4
208 days in a dark cupboard at ambient temperature (21⁰C). Texture profile analysis of the cake
209 crumb was carried out at 0, 1, 2 and 4 days of storage using a texture analyser (CT3, Brookefield
210 Viscometers Ltd, Harlow, UK) equipped with a cylinder probe (TA25/1000, D=25.4mm). Sample
211 cubes (20 mm x 20 mm x 20 mm) were prepared from the cake slices (in triplicate). The cubes
212 were 50% compressed twice to give a two bite texture profile. Trigger load and test speed were
213 5 g and 0.5 mm/s respectively.

214

215 Statistical analysis

216 Statistical analysis was carried out using SPSS (version 22, IBM, Portsmouth, UK), and data
217 processed using MS Excel software (Microsoft, Reading, UK). Total TBARS and PC formed during
218 four hours of in vitro gastric digestion and two hours of intestinal digestion were quantified by
219 calculating the Areas Under the digestion Curves (AUC) using the trapezoidal rule. Data on the

220 TBARS and PC contents in the cakes, AUCs from gastrointestinal digestions, antioxidant capacity,
221 total polyphenol content and Rancimat induction times were analysed using one-way ANOVA
222 with cake type and parameters as the independent and dependent factors respectively. Texture
223 data was analysed using a factorial ANOVA model. *Post hoc* tests were carried out using the
224 Tukey, Ryan, Einot, Gabriel and Welsch Q procedure, and Dunnett's test as appropriate. A $p < 0.05$
225 was considered significant. Data normality was assessed using the Kolmogorov-Smirnov test.

226

227 **Results and Discussion**

228

229 Similar to chocolate the high prevalence of natural antioxidants in beetroot is well documented
230 (Clifford et al. 2015). To our knowledge this is the first study comparing beetroot and chocolate
231 and their combined effects on antioxidant and functional properties, particularly within a
232 processed food model that is inherently high in fat and protein, and therefore prone to oxidation.
233 Compositional analysis of the cakes using dietary software (NetWisp, Tinuviel Software,
234 Warrington, UK) indicated that addition of beetroot did not alter macronutrient contents (Table
235 2) but increased total fibre and micronutrient contents, particularly Potassium, Phosphorus, Iron
236 and folate. The improvement in nutritional properties is unsurprising as beetroot and cocoa are
237 rich in fibre, micronutrients and trace elements (Ninfali and Angelino 2013, Steinberg et al. 2003).

238

239 Total antioxidant potential and polyphenol content of cakes

240 In agreement with previous observations (Li et al. 2015) the antioxidant potential of cakes
241 showed a strong correlation with polyphenol contents ($p < 0.001$; $r = .97$). There was a significant

242 effect of cake type on the antioxidant capacity ($F(3, 8) = 873.38, p < 0.001$), and post hoc analyses
243 showed that the CB cake had a significantly higher antioxidant capacity ($22.6 \pm 1.0 \mu\text{M Fe(II)}/\text{mL}$)
244 when compared with the other three cakes (PN: $7.6 \pm 0.4 \mu\text{M Fe(II)}/\text{mL}$, PB: $11.8 \pm 1.1 \mu\text{M Fe(II)}/$
245 mL and CN: $15.4 \pm 1.2 \mu\text{M Fe(II)}/\text{mL}$) (Figure 1). This indicates the presence of both these natural
246 products had cumulative effects on antioxidant status. The addition of beetroot (0.24g/g) or
247 cocoa (0.05 g/g) increased the antioxidant potential of the cake to similar degrees supporting
248 evidence that cocoa has a greater antioxidant potential on a per-gram basis (Belščak et al. 2009,
249 Wootton-Beard et al. 2011). The advantage of beetroot however is its bulkiness which could
250 replace fat and carbohydrate ingredients whilst conferring antioxidant levels comparable to
251 cocoa.

252
253 A significant effect of cake type on total polyphenol content was also observed ($F(3, 8) = 278.5,$
254 $p < 0.001$) (Figure 1) with the four cakes showing significantly different levels. The chocolate cakes
255 (CB and CN) had significantly higher levels of polyphenols (CB: $574.9 \pm 12.3 \mu\text{g GAE/g}$ of sample,
256 CN: $410.7 \pm 16.5 \mu\text{g GAE/g}$ of sample) when compared to the cakes without chocolate (PB: 334.7
257 $\pm 9.6 \mu\text{g GAE/g}$ of sample, PN: $303.1 \pm 11.3 \mu\text{g GAE/g}$ of sample), and this further supports
258 chocolate as a rich source of phytochemicals. No additive effects on antioxidant potential or total
259 polyphenols were observed when chocolate and beetroot were combined, which may be due to
260 masking of some polyphenols with proteins, carbohydrates and fats (Jakobek 2015). However,
261 published data on this is equivocal as the degree of binding may depend on factors such as
262 polyphenol chemistry, matrix, macronutrient characteristics and processing conditions. For

263 instance, heating has been shown to increase the binding of polyphenols (Yazdi and Corredig
264 2012).

265

266 Oxidative stability of cakes

267 Reflecting antioxidant potential and total polyphenols, Rancimat determined induction times
268 were also significantly affected by cake type ($F(3,8)=88.8$; $P<0.001$) (Figure 2), where that of CB
269 cake was almost three-fold longer (43.4 ± 1.2 hours) than PN cake (15.5 ± 0.3 hours), suggesting
270 strong synergistic effects. Induction times for CN (25.6 ± 0.4) and PB (24.0 ± 4.1) cakes did not
271 significantly differ ($P>0.05$) indicating both these ingredients had comparable effects on product
272 shelf life. However, their combination in the cake served to markedly increase oxidative stability
273 as evidenced by the absolute increment seen in CB compared to PN (27.9 hours) which was over
274 two-fold higher than was seen for CN and PB (10.1 and 8.5 hours respectively)

275

276 Prolongation of induction times of cake with beetroot addition agrees with our previous findings
277 with mayonnaise and bread (Raikos et al. 2015, Ranawana et al. 2016a). The present study
278 indicated that comparable effects on oxidative stability occur with addition of chocolate, and this
279 has not been previously reported. Induction time is a predictor of product shelf life (Farhoosh
280 2007) which suggest that the addition of beetroot and chocolate increases product longevity,
281 possibly through the antioxidant effects of the inherent phytochemicals. Inclusion of these
282 natural ingredients could allow reduction in usage of adversely perceived synthetic antioxidants
283 (Shahidi and Ambigaipalan 2015) allowing manufacturers to limit problematical lipid and protein
284 oxidation of commercially processed products while enhancing nutritional benefits.

285

286 Generation of thiobarbituric acid reactive substances (TBARS) during digestion

287 There was a significant effect of cake type on the total amount of TBARS generated during the
288 gastric phase of digestion ($F(3, 20) = 24.5, p < 0.001$) (Figure 3), and post hoc analyses showed
289 that the PN cake contained a significantly higher amount of TBARS (75819.9 ± 1605.0 nmol/g.min,
290 $p < 0.001$) when compared to the CN (49689.7 ± 6431.9 nmol/g.min) and CB cakes ($53988.6 \pm$
291 7820.3 nmol/g.min), suggesting the addition of chocolate and beetroot reduced fat oxidation
292 during this phase. The intestinal phase showed a general increase in the amount of TBARS
293 produced in the chocolate cakes (CB and CN) when compared to the gastric phase, and showed
294 significant differences ($F(3, 20) = 3.69, p < 0.05$). The CB and PN cakes showed similar levels of
295 TBARS (44875.8 ± 2971.2 nmol/g.min and 40356.9 ± 4357.2 nmol/g.min respectively), however
296 the former contained a significantly higher amount compared to the CN and PB cakes.

297

298 The study provides a first comparative record of the effects of beetroot and cocoa addition on
299 oxidation of macronutrients contained in a processed food during gastrointestinal digestion. The
300 human alimentary tract can often be oxygen-rich showing gradients along its length and breadth
301 (Espey 2013) and this could be exacerbated by mastication which aerates the chyme. Therefore,
302 protecting macronutrients from oxidation during digestion could be an important role of dietary
303 antioxidants. Chocolate and beetroot polyphenols have been shown to be stable during gastric
304 transit (Rios et al. 2002, Wootton-Beard et al. 2011) suggesting their antioxidant properties
305 should remain intact. We found that the chocolate-containing treatments lowered lipid oxidation
306 (measured as TBARS) during the gastric phase and had equivocal effects during the intestinal

307 phase and this agrees with evidence showing cocoa polyphenols are more stable in acidic pH
308 (Andres-Lacueva et al. 2008). Beetroot did not curtail lipid oxidation during digestion and this too
309 agrees with previous observations (Ranawana et al. 2016a). Interestingly however, the
310 combination of beetroot and cocoa significantly increased TBARS in the intestinal phase
311 compared to when they were alone. We are unable to propose a reason for this observed
312 negative additive effect and warrants further study.

313

314 Generation of protein carbonyls during digestion

315 Protein carbonyls are formed during the oxidative cleavage of proteins, from the production of
316 carbonyl groups during protein oxidation, and as secondary reactions of lipid oxidation.
317 Therefore, the PC composition in a food would depend on the quality and quantity of
318 macronutrients contained in it. Their relative stability makes them a useful measure of protein
319 oxidation.

320

321 The amount of PCs observed during the gastric phase of digestion was significant ($F(3, 8) = 17.08$,
322 $p = 0.001$), and post hoc analyses showed that the chocolate cakes (CB: 130009.18 ± 29587.05
323 pmol/g.min and CN: $116711 \pm 29372.07 \text{ pmol/g.min}$) contained significantly higher amounts than
324 the others (PB: 30856.0 ± 11521.2 and PN: $30432.14 \pm 12735.14 \text{ pmol/g.min}$) (Figure 3). Similarly,
325 protein carbonyls in the intestinal phase was significant ($F(3, 8) = 16.88$, $p = 0.001$) with the two
326 chocolate-containing cakes produced similar but higher levels compared to the PB and PN cakes.
327 The higher carbonyl levels at all digestion-phases for the chocolate-containing cakes suggest they
328 originated from the cocoa, and indeed carbonyls are abundant in cocoa representing an

329 important flavour group (Aprotosoie et al. 2016). Carbonyls are a diverse family of compounds
330 comprising both beneficial (flavour) and harmful (food degradation and oxidative stress) variants
331 (Fedorova et al. 2014) and our method was unable to distinguish between them. Therefore it
332 remains to be confirmed how the addition of cocoa may be affecting undesirable protein
333 oxidation during digestion. In agreement with previous observations beetroot did not impact on
334 PC generation at any of the digestion phases (Ranawana et al. 2016a).

335

336 Textural changes during storage of cakes

337 Texture has been shown to be affected by oxidation, and the addition of natural products rich in
338 phytochemicals have been demonstrated to curtail related processes such as retrogradation
339 (Patrignani et al. 2014). Therefore, texture was analysed as part of the study to assess how
340 reformulation with beetroot and chocolate affected this physical attribute. The four cake types
341 had significantly different levels of hardness with an interactive effect of treatment and day (F
342 $(15, 32) = 4.30, p = 0.001$) (Table 3). All four cakes increased in hardness during storage, being
343 similar on day 1 but increasing by day 2. *Post hoc* analyses showed the hardness of CB, CN and
344 PB cakes initially were less than PN cake but these differences were not apparent by day 4. This
345 suggests that the addition of chocolate and beetroot has beneficial short-term effects on
346 hardness. Significant differences in the degree of adhesiveness were also observed between the
347 four cake types but there were no discernible interactive effects of treatment and day.
348 Adhesiveness tended to fluctuate in the cakes during storage with no discernible pattern (Table
349 3) although the PB cake showed the highest degree of adhesiveness overall (0.825 ± 0.4). This

350 may be due to the greater water retention capacity of beetroot (Shyamala and Jamuna 2010),
351 suggesting it could be used to improve the moistness of products.

352
353 Fracturability was similar in all the cakes when fresh (day 0) and showed significant increases
354 after 1, 2 and 4 days of storage ($p < 0.001$) (Table 3) with PN cake showing the greatest values by
355 days 2 and 4. Therefore beetroot and chocolate appear to reduce fracturability during storage,
356 which is desirable in baked products. Notably, the CN cake showed the overall lowest
357 fracturability during storage. Springiness was significantly different in the four cake types when
358 fresh ($(F(3, 8) = 13.4, p = 0.002)$) with *post hoc* tests showing CN and PB cakes having significantly
359 higher values than PN cake. However, all four cakes showed comparable springiness after 1, 2
360 and 4 days of storage. Overall, beetroot did not adversely alter the texture of cake and this agrees
361 with previous data using bread (Ranawana et al. 2016a). Combining beetroot and chocolate does
362 not appear to have adverse effects on textural parameters and this is promising from a sensory
363 perspective.

364
365 Phytochemical stability during processing

366 The predominant phytochemicals in beetroot include betalains, ferulic acid derivatives, phenolic
367 amides and flavonoids (Kujala et al. 2002) whilst cocoa contains a more complex mixture of
368 catechins, procyanidins, anthocyanins and flavonols (Wollgast and Anklam 2000). Processing
369 conditions have been shown to affect the antioxidant properties of phytochemicals (Kalt 2005)
370 highlighting the need to assess their oxidative effects on a product-specific basis. In sponge cake
371 baking core temperatures usually do not exceed 100°C (Fehaili et al. 2010). Cocoa polyphenols

372 have a relatively high thermal stability as they have withstood roasting temperatures around
373 150°C (Ramli et al. 2006), and therefore would be stable within the cake matrix. The beetroot
374 used in the cakes was microwaved as our work showed that mild heat processing improves
375 betalain stability (Raikos et al. 2016). However, the temperature-time combination may be
376 important for phytochemical stability.

377

378 The thermal treatment of betalain produces degradation products such as isobetanin and
379 neobetanin which are found in high quantities in processed beetroot products (Herbach et al.
380 2004). Although we did not measure betalain degradation products in the cakes it is likely they
381 were high due to the two thermal treatments the beetroot was subjected to. Limited work has
382 been carried out to determine the functional properties of these degradation products. Wootton-
383 Beard et al (2014) found that the consumption of a beetroot juice predominating in neobetalins
384 significantly reduced glycaemic and insulinaemic responses in volunteers, suggesting they may
385 have functional properties. This study showed that the cooked beetroot cakes had high
386 antioxidant potentials which is suggestive of antioxidant effects of heat degraded betalains.
387 However this remains to be confirmed in future studies.

388

389 **Conclusion**

390 To our knowledge this is the first study assessing the singular and combined effects of beetroot
391 and chocolate addition on oxidative stability of a processed food, both in the product and during
392 simulated gastro-intestinal digestion. In response to consumer and public health demands, as
393 protein and unsaturated fat contents increase in processed foods so does the importance of

394 antioxidant ingredients for protecting them. The present study showed that beetroot increased
395 the antioxidant and polyphenol profiles of sponge cake which further improved with the addition
396 of chocolate. Beetroot also improved the oxidative stability and estimated shelf-life of sponge
397 cake, and these effects were further enhanced when combined with chocolate. Chocolate was
398 more promising in curtailing lipid oxidation during gastro-intestinal digestion while beetroot
399 showed neutral effects on both lipid oxidation and protein oxidation. Textural parameters were
400 not adversely affected by beetroot and chocolate addition, and both slowed staling suggesting
401 positive effects. Overall, the results indicated that the benefits of beetroot and chocolate
402 addition were manifested more in the food system through improving oxidative stability and
403 shelf life, than during its digestion. However, their presence did not adversely affect
404 macronutrient oxidation during digestion but served to marginally improve protection.

405

406 **Acknowledgments**

407 The authors are grateful to the Rural and Environment Science and Analytical Services (RESAS)
408 Division of the Scottish government for funding the study. None of the authors declare any
409 conflicts of interest.

410

411

412

413

414

415

416 **References**

- 417 Andres-Lacueva C, Monagas M, Khan N, Izquierdo-Pulido M, Urpi-Sarda M, Permanyer J,
418 Lamuela-Raventos R (2008) Flavanol and flavonol contents of cocoa powder products: influence
419 of the manufacturing process. *J Agric Food Chem* 56:3111-3117.
- 420 Aprotosoiaie AC, Luca SV, Miron A (2016) Flavor chemistry of cocoa and cocoa products—an
421 overview. *Compr Rev Food Sci Food Saf* 15:73-91.
- 422 Belščak A, Komes D, Horžić D, Ganić KK, Karlović D (2009) Comparative study of commercially
423 available cocoa products in terms of their bioactive composition. *Food Res Int* 42:707-716.
- 424 Campbell L, Euston SR, Ahmed MA (2016) Effect of addition of thermally modified cowpea
425 protein on sensory acceptability and textural properties of wheat bread and sponge cake. *Food*
426 *Chem* 194:1230-1237.
- 427 Clifford T, Howatson G, West DJ, Stevenson EJ (2015) The potential benefits of red beetroot
428 supplementation in health and disease. *Nutrients* 7:2801-2822.
- 429 Duthie G, Campbell F, Bestwick C, Stephen S, Russell W (2013) Antioxidant effectiveness of
430 vegetable powders on the lipid and protein oxidative stability of cooked turkey meat patties:
431 implications for health. *Nutrients* 5:1241-1252.
- 432 Espey MG (2013) Role of oxygen gradients in shaping redox relationships between the human
433 intestine and its microbiota. *Free Rad Biol Med* 55:130-140.
- 434 Farhoosh R (2007) The effect of operational parameters of the Rancimat method on the
435 determination of the oxidative stability measures and shelf-life prediction of soybean oil. *J Am*
436 *Oil Chem Soc* 84:205-209.
- 437 Fedorova M, Bollineni RC, Hoffmann R (2014) Protein carbonylation as a major hallmark of
438 oxidative damage: update of analytical strategies. *Mass Spectrom Rev* 33:79-97.
- 439 Fehaili S, Courel M, Rega B, Giampaoli P (2010) An instrumented oven for the monitoring of
440 thermal reactions during the baking of sponge cake. *J Food Eng* 101:253-263.
- 441 Goya L, Martín MÁ, Sarriá B, Ramos S, Mateos R, Bravo L (2016) Effect of cocoa and its
442 flavonoids on biomarkers of inflammation: studies of cell culture, animals and humans.
443 *Nutrients* 8:212-224.
- 444 Herbach K, Stintzing F, Carle R (2004) Impact of thermal treatment on color and pigment
445 pattern of red beet (*Beta vulgaris* L.) preparations. *J Food Sci* 69:492-498.

446 Jakobek L (2015) Interactions of polyphenols with carbohydrates, lipids and proteins. Food
447 Chem 175:556-567.

448 Kalt W (2005) Effects of production and processing factors on major fruit and vegetable
449 antioxidants. J Food Sci 70:11-19.

450 Kujala TS, Vienola MS, Klika KD, Loponen JM, Pihlaja K (2002) Betalain and phenolic
451 compositions of four beetroot (*Beta vulgaris*) cultivars. Eur Food Res Technol 214:505-510.

452 Leroy P, Requillart V, Soler L, Enderli G (2015) An assessment of the potential health impacts of
453 food reformulation. Eur J Clin Nutr:694-699.

454 Li X, Wasila H, Liu L, Yuan T, Gao Z, Zhao B, Ahmad I (2015) Physicochemical characteristics,
455 polyphenol compositions and antioxidant potential of pomegranate juices from 10 Chinese
456 cultivars and the environmental factors analysis. Food Chem 175:575-584.

457 Miyata T, Inagi R, Asahi K, Yamada Y, Horie K, Sakai H, Uchida K, Kurokawa K (1998) Generation
458 of protein carbonyls by glycooxidation and lipoxidation reactions with autoxidation products of
459 ascorbic acid and polyunsaturated fatty acids. FEBS Lett 437:24-28.

460 Niedernhofer LJ, Daniels JS, Rouzer CA, Greene RE, Marnett LJ (2003) Malondialdehyde, a
461 product of lipid peroxidation, is mutagenic in human cells. J Biol Chem 278:31426-31433.

462 Ninfali P, Angelino D (2013) Nutritional and functional potential of *Beta vulgaris* cicla and rubra.
463 Fitoterapia 89:188-199.

464 Patrignani M, Conforti PA, Lupano CE (2014) The role of lipid oxidation on biscuit texture during
465 storage. Int J Food Sci Tech 49:1925-1931.

466 Raikos V, McDonagh A, Ranawana V, Duthie G (2016) Processed beetroot (*Beta vulgaris* L.) as a
467 natural antioxidant in mayonnaise: Effects on physical stability, texture and sensory attributes.
468 Food Sci Hum Wellness 5:191-198.

469 Raikos V, Neacsu M, Morrice P, Duthie G (2015) Anti-and pro-oxidative effect of fresh and
470 freeze-dried vegetables during storage of mayonnaise. J Food Sci Tech:1-10.

471 Ramli N, Hassan O, Said M, Samsudin W, Idris NA (2006) Influence of roasting conditions on
472 volatile flavor of roasted Malaysian cocoa beans. J Food Process Preserv 30:280-298.

473 Ranawana DV, Raikos V, Campbell F, Bestwick C, Nicol P, Milne L, Duthie G (2016a) Breads
474 Fortified with Freeze-Dried Vegetables: Quality and Nutritional Attributes. Part 1: Breads
475 Containing Oil as an Ingredient. Foods 5:19-32.

476 Ranawana V, Campbell F, Bestwick C, Nicol P, Milne L, Duthie G, Raikos V (2016b) Breads
477 Fortified with Freeze-Dried Vegetables: Quality and Nutritional Attributes. Part II: Breads Not
478 Containing Oil as an Ingredient. *Foods* 5:62-76.

479 Rios LY, Bennett RN, Lazarus SA, Remesy C, Scalbert A, Williamson G (2002) Cocoa procyanidins
480 are stable during gastric transit in humans. *Am J Clin Nutr* 76:1106-1110.

481 Santos M, Coe S (2016) The total polyphenol content of various commercial cocoa beverages,
482 with and without the addition of cow's milk. *Proc Nutr Soc* 75.

483 Shahidi F, Ambigaipalan P (2015) Phenolics and polyphenolics in foods, beverages and spices:
484 Antioxidant activity and health effects—A review. *J funct foods* 18:820-897.

485 Shyamala B, Jamuna P (2010) Nutritional Content and Antioxidant Properties of Pulp Waste
486 from *Daucus carota* and *Beta vulgaris*. *Malays J Nutr* 16:397-408.

487 Steinberg FM, Bearden MM, Keen CL (2003) Cocoa and chocolate flavonoids: implications for
488 cardiovascular health. *J Am Diet Assoc* 103:215-223.

489 Wollgast J, Anklam E (2000) Review on polyphenols in *Theobroma cacao*: changes in
490 composition during the manufacture of chocolate and methodology for identification and
491 quantification. *Food Res Int* 33:423-447.

492 Wootton-Beard PC, Brandt K, Fell D, Warner S, Ryan L (2014) Effects of a beetroot juice with
493 high neobetanin content on the early-phase insulin response in healthy volunteers. *J Nutr Sci*
494 3:1-9.

495 Wootton-Beard PC, Moran A, Ryan L (2011) Stability of the total antioxidant capacity and total
496 polyphenol content of 23 commercially available vegetable juices before and after in vitro
497 digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Res Int* 44:217-
498 224.

499 Yazdi SR, Corredig M (2012) Heating of milk alters the binding of curcumin to casein micelles. A
500 fluorescence spectroscopy study. *Food Chem* 132:1143-1149.

501
502
503
504
505
506
507
508
509

510 **Legends to figures**

511

512 **Figure 1:** Antioxidant capacity and polyphenol content of cakes. CB: Chocolate and beetroot
513 cake, CN: Chocolate cake, PB: Beetroot cake, PN: Plain cake. Columns with different letters are
514 significantly different, One-way ANOVA, $p < 0.05$. Error bars are standard errors.

515

516 **Figure 2:** Oxidative stability of cake measured as Induction time. Columns with different letters
517 are significantly different, One-way ANOVA, $p < 0.05$. Error bars are standard errors.

518

519 **Figure 3:** Changes in Thiobarbituric acid reactive substances (TBARS) (A) and Protein Carbonyls
520 (B) in cakes during simulated gastro-intestinal digestion. Solid lines represent the baseline, oral
521 and gastric phases, the broken lines represent the small intestinal phase. CB: Chocolate and
522 beetroot cake, CN: Chocolate cake, PB: Beetroot cake, PN: Plain cake. Error bars are standard
523 errors.

524

525

526

527

528

529

530

531

532

533

534

535

536

537

538

539

540 **Tables**

541

542 **Table 1:** Ingredient composition of cakes

Ingredients (g)	Source	Chocolate	Chocolate	Plain	Plain cake
		cake with beetroot	cake without beetroot	cake with beetroot	without beetroot
Cocoa powder	Cadbury Bourneville	50	50	-	-
All-purpose flour	Tesco stores	175	225	225	275
Baking powder	Dr Oetker	9	9	9	9
Caster sugar	Silver spoon	200	200	200	200
Beetroot	Sainsbury's	250	-	250	-
	supermarkets, var. Globe				
Eggs	Tesco stores	130	130	130	130
Rapeseed oil	Tesco organic	225	225	225	225
Salt	Saxa	3	3	3	3
Water	-	0	200	0	200
Total batter		1042	1042	1042	1042
weight					
Weight reduction		9.3	9.6	9.1	10.0
after baking (%)					

543

544

545

546

547 **Table 2:** Nutrition composition of the cakes

Nutrient	CB cake	CN cake	PB cake	PN cake
Protein (g)	4.6	4.5	4.2	4.1
Fat (g)	24.3	24.3	23.3	23.3
Carbohydrate (g)	36.3	37.8	39.5	40.9
AOAC fibre (g)	2.8	2.5	1.4	1.1
Potassium (mg)	237	122	172	58
Magnesium (mg)	34	31	10	8
Phosphorus (mg)	176	161	150	135
Iron (µg)	1.33	1.23	0.92	0.82
Folate (µg)	19	6	19	6
Water content (%)	21.3	20.5	18.9	18.5

548 CB: Chocolate beetroot cake, CN: Plain chocolate cake, PB: Beetroot cake, PN: Plain cake. Compositions
 549 determined using nutritional analysis software NetWisp, Tinuviel Software, Warrington, UK.

550

551

552

553

554

555

556

557

558

559 **Table 3:** Textural parameters of cakes when fresh and during storage

Day 0	Hardness (g) (cycle 1)	Hardness (g) (cycle 2)	Adhesiveness (mJ)	Fracturability (g)	Springiness (mm)
CB	397.3±103.2	306.7±91.1	0.16±0.1	397.3±103.2	8.8±0.5 ^{ab}
CN	218.7±27.4	166.7±23.1	0.36±0.2	179.3±47.7	10.1±0.4 ^c
PB	391.7±94.1	320.0±64.2	0.90±0.5	391.7±94.1	9.3±0.1 ^{bc}
PN	417.7±108.6	308.3±72.5	0.46±0.1	417.7±108.6	8.2±0.4 ^a
Day 1					
CB	546.3±33.9	403.0±25.2	0.26±0.2	546.3±33.9	9.0±0.3
CN	464.7±131.2	335.3±112.6	0.13±0.1	464.7±131.2	9.8±0.9
PB	359.7±52.0	294.0±31.8	0.46±0.1	359.7±52.0	10.0±0.5
PN	555.0±133.1	431.3±95.8	0.60±0.5	521.7±183.2	9.0±1.0
Day 2					
CB	598.7±37.4 ^{bc}	445.3±33.5 ^{bc}	0.26±0.3	598.66±37.4 ^{bc}	9.1±0.2
CN	376.3±25.6 ^a	267.3±12.7 ^a	0.43±0.3	376.33±25.6 ^a	8.6±1.0
PB	500.0±47.6 ^{ac}	386.3±30.9 ^{ac}	1.13±0.6	500.0±47.6 ^{ac}	10.0±0.4
PN	1081.0±158.0 ^d	751.3±95.3 ^d	0.40±0.4	1081.0±158.0 ^d	8.1±0.4
Day 4					
CB	723.0±280.1	516.6±208.7	0.76±0.7	723.0±280.1	8.98±0.8
CN	501.3±28.2	339.7±24.6	0.40±0.2	446.0±47.9	7.9±0.5
PB	624.3±80.7	473.3±63.5	0.80±0.3	624.3±80.7	8.6±0.2
PN	844.7±22.5	601.3±13.2	0.70±0.26	787.3±97.2	7.6±0.5

560 Day 0 represents fresh cakes; Values are means ± Standard Deviations; CB: Chocolate beetroot cake, CN: Plain Chocolate cake,
 561 PB: Plain beetroot cake, PN: Plain cake; Values within a column for each day with different superscript letters are significantly
 562 different, One-way ANOVA, p<0.05. Columns with no superscripts denote statistically similar values for the four cakes.