Proximate composition, functional properties and quantitative analysis of benzoyl peroxide and benzoic acid in wheat flour samples: effect on wheat flour quality

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Background. Extensive milling processes have deprived wheat flour from essential nutrients. The objective of the current study was to assess the nutritive quality of commercial wheat flour (soft flour/SF) through analyses of proximate composition and functional properties as well as quantification of benzoyl peroxide (BPO; added as bleaching agent in the SF) by comparing the results with whole wheat flour (WF; never received any additives). Methods. The samples included commercial soft flour (SF) purchased from the local supplier of different flour mills (who use BPO as additive) and a control sample without additives was prepared by grinding the seeds harvested from wheat (*Triticum aestivum* L.; Inqulab 91) crop grown in the experimental field of University of Agriculture, Faisalabad, under optimized field conditions without any fertilizers and insecticides. Functional properties (including bulk density, water absorption capacity, oil absorption capacity, emulsifying activity, foaming capacity, least gelatinization concentration and gelatinization temperature) and proximate composition (including moisture content, ash contents, crude protein, gluten and starch contents) were determined and compared for all the samples. Benzoyl peroxide (BPO) and Benzoic Acid (BA) quantification was performed through High Performance Liquid Chromatography (HPLC). Finally dietary intake was estimated for BPO and BA. Results. Results showed that SF had lesser fiber, protein and ash contents, whereas, higher damaged starch, fat, gluten and bulk density. A parallel experiment under selected conditions (temperature, time and solute concentration) showed dissociation of BPO into BA soon after the exposure. Observed BA range (13.77 mg/g after 16h) in SF and exposure level assessment (44.3±1.36 mg/kg/BW) showed higher intake of BA on the consumption of SF. Results

revealed superiority of WF over SF in nutritive qualities as well as free of toxicants such as BA.

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Abstract

Background.

Extensive milling processes have deprived wheat flour from essential nutrients. The objective of the current study was to assess the nutritive quality of commercial wheat flour (soft flour/SF) through analyses of proximate composition and functional properties as well as quantification of benzoyl peroxide (BPO; added as bleaching agent in the SF) by comparing the results with whole wheat flour (WF; never received any additives).

Methods.

The samples included commercial soft flour (SF) purchased from the local supplier of different flour mills (who use BPO as additive) and a control sample without additives was prepared by grinding the seeds harvested from wheat (*Triticum aestivum* L.; Inqulab 91) crop grown in the experimental field of University of Agriculture, Faisalabad, under optimized field conditions without any fertilizers and insecticides. Functional properties (including bulk density, water absorption capacity, oil absorption capacity, emulsifying activity, foaming capacity, least gelatinization concentration and gelatinization temperature) and proximate composition (including moisture content, ash contents, crude protein, gluten and starch contents) were determined and compared for all the samples. Benzoyl peroxide (BPO) and Benzoic Acid (BA) quantification was performed through High Performance Liquid Chromatography (HPLC). Finally dietary intake was estimated for BPO and BA.

Results.

Results showed that SF had lesser fiber, protein and ash contents, whereas, higher damaged starch, fat, gluten and bulk density. A parallel experiment under selected conditions (temperature, time and solute concentration) showed dissociation of BPO into BA soon after the exposure. Observed BA range (13.77 mg/g after 16h) in SF and exposure level assessment (44.3±1.36 mg/kg/BW) showed higher intake of BA on the consumption of SF. Results revealed superiority of WF over SF in nutritive qualities as well as free of toxicants such as BA.

KEYWORDS: *Benzoyl peroxide*; *Benzoic acid; Soft Flour*; *Whole Wheat Flour*; *High Performance Liquid Chromatography*

Introduction

58

59   Wheat (*Triticum aestivum* L.) is a principal cereal consumed world-wide in different forms, as a

60   major food source. During milling process the grains are milled to obtain flour which is pale yellow

61   in colour. The bakery products it yields are less commercially acceptable, therefore flour is

62   bleached for different bakery items. When this flour is stored, natural maturation or aging takes

63   place, where carotenoids undergo oxidation and improves rheological properties of the dough such

64   as texture (Liu et al. 2014). Lutein (a xanthophyll) is excessively found in the wheat seeds

65   ($\approx$3mg/kg of dried wheat) and the yellow colour of the bread is due to the remains of lutein in

66   wheat seeds (Mellado-Ortega & Hornero-Méndez 2017).

67   Wheat milling industries utilize oxidizing agents such as benzoyl peroxide (BPO) to accelerate the

68   process of maturation and dough improvement. These agents oxidize sulf-hydryl groups in flour

69   gluten protein that yields increased stickiness of the flour and makes its appearance as soft flour

70   (SF) (Onishi et al. 2004b). These oxidizing agents can be used for bleaching and dough

71   improvement. During flour bleaching conjugated double bond of carotenoids is disrupted to less

72   conjugated colorless system, which gives flour a desirable texture for baking. One of the most

73   commonly used oxidizing agent is BPO which exhibits bleaching properties without influencing

74   the baking or taste (Gaddipati et al. 1983). BPO is a free radical initiator and it produces carotenoid

75   oxidation like a typical free radical mechanism. No acceptable limits of BPO have been specified

76   in the food safety regulations. BPO has been extensively used throughout the world as a bleaching

77   agent without any recovery. In food processing BPO is (> 92%) converted into benzoic acid (BA),

78   which is usually used as a preservative for soft drinks, fruits, juices and many other types of food

79   items. The higher concentration of BA than allowable safety level (40 mg/kg) is harmful for

80   humans. Acute toxicity is unknown, however, a sensitive person consuming BA more than 5 mg/kg

81   of body weight (BW) per day can initiate non-immunological (pseudo-allergy) reactions,

82   hyperpnoea, metabolic acidosis and convulsions (Wei et al. 2006; Liu 2007).

83   Beside addition of BPO, the SF is processed several times compared to the WF to achieve the fiber

84   free fine particles for the improved texture of the bakery products. During this processing structure,

85   sensory qualities, protein contents, fiber contents and nutritional components get severely affected.

86   Antioxidants in WF, which are present in the germ and bran (Isabelle & Andre 2006), are mostly

87   removed in the refined flour. Therefore, the current study was designed to observe and compare

88   the proximate composition and functional properties of SF and WF as well as BPO and BA

89   concentrations in them. Current study also considered the conversion of BPO to BA at different

90   time intervals after adding a known concentration of BPO to WF (control; without additives;

91   grown under similar conditions to control with additives). The findings will help to identify the

92   levels of BA in SF which may get consumed and nutritional deprivation as a result of intensive

93   grinding and sieving of wheat flour during milling.

Materials & Methods

94

Sample collection

95

96   Test samples included commercial soft flour (n=4, SF) purchased from the local supplier from

97   different flour mills (with additives) and a control sample (n=1, WF) without additives was

98   prepared by mechanical grinding the *Triticum aestivum L.* seeds (Inqulab 19), harvested from the

99   crop grown on the experimental field of University of Agriculture, Faisalabad, under optimized

100 field conditions (16/8 D/N; 23±1°C; 14 Inch water) without any fertilizer and insecticide. The

101 variety of wheat seeds and growth conditions were kept same as used by flour mills to generate

102 SF, to avoid ambiguity in results. The WF flour sample was passed through sieve (75µm size)

103 before packing them into air-tight plastic containers. The size was in agreement with the particle

104 size of SF purchased from flour mills.

Determination of functional properties

105

106 Bulk density of flour was determined as described previously (Jehu-Appiah et al. 2011) by

107 following ASTM D1895B prescribed procedure. The sample was allowed to flow freely in a

108 circular container (0.615 L) with a suspended funnel of opening diameter (1.5 cm). The height of

109 funnel was kept about 20 cm and the powder was stirred continuously to avoid clogging inside the

110 opening. Container with the sample was dropped few times from the height of 150 mm to allow

111 settling and release of air. Weight of the container with the sample was determined and

112 weight/volume (loose bulk density) was obtained. Density was determined through the formula,

113 $d = \frac{m}{v}$, where mass (*g*) is the weight of the sample and volume (mL) is the volume of the material.

114 Water absorption capacity and oil absorption capacity (WAC and OAC respectively) were

115 determined through the method described by Beuchat (1977). In this method, 1g of sample was

116 allowed to mix with 10mL of distilled water for about 30 sec. Sample was then allowed to stand

117   at room temperature ($25 \pm 2\,°C$) for the next 30 min and centrifuged at 3000 rpm (30 min). Volume

118   of the supernatant was determined and WAC (mg/mL) was calculated by formula $WAC = V_{initial}$

119    $- V_{final}$, where V is the volume of water (mL). Similar procedure was repeated for OAC

120   determination using soybean oil (Sp. Gravity 0.9092) as the absorbing agent. Emulsifying stability

121   and emulsifying activity (ES and EA respectively) were determined by the method described by

122   Neto et al. (2001) with modifications. About 5 mL of flour dispersion (10 mg/mL of water) was

123   homogenized with 5 mL of soybean oil for 1 min through vigorous shaking. The emulsion was

124   then centrifuged (Sigma 2-6, Germany) at 1100 rpm for 10 min. Height (cm) of emulsified layer

125   (ELH) was deducted from the total height of the tube contents (TC) to estimate the EA by the

126   equation $EA = (\frac{ELH}{TC}) \times 100$. ES was calculated by heating the emulsion at 80°C for 30 min before

127   centrifuging at 1300 rpm for 10 min. ES was then calculated by $ES = (\frac{ELHA}{TCA}) \times 100$, where ELHA

128   is the height of emulsified layer after heating and TCA is total content of the tube before heating.

129   The effect of EA and ES was determined by varying the capacity of the flour samples. Foaming

130   stability and capacity (FS and FC) of flours were determined by the method reported by Coffmann

131   & Garciaj (1977), where about 100 mL of distilled water was mixed with 10 g of flour. The

132   suspension was mixed vigorously for 5 min on the magnetic stirrer (Irmco MSC Digital,

133   Germany). The initial solution volume V_1 and the final solution volume V_2 were recorded (using

134   graduated cylinder). Foaming Capacity (FC) was also calculated from the formula $FC = (\frac{V_2 - V_1}{V_1}$

135    $) \times 100$. Foaming Stability (FS) was also determined by the foam volume that was left for 8h and

136   expressed as percentage of initial foam volume. To determine the gelation properties; Least

137   gelatinization concentration (LGC) and gelatinization temperature (GnT), distilled water sample

138   suspensions (2-10% w/v) were prepared. About 10 mL of these suspensions were transferred into

139   the test tubes. In the boiling water bath these test tubes were heated for 1h and then cooled for 2h

140   at 4°C in a refrigerator. Least gelation concentrations were taken when the samples did not fall

141   from the inverted test tubes.

142   ## Proximate composition

143   Proximate composition was determined through FT-NIR spectroscopy (Burker-TENSOR 37 FTIR

144   spectrometer, Germany) as well as conventional methods to get reliable results. These properties

145   included moisture content (MC), ash contents (AC), crude protein (CP), gluten and starch contents.

146   Other properties such as crude fiber and fat (CFF) were determined through solvent extractions.

147   For crude fiber about 2.5-3g of sample was transferred to Soxhlet and extracted with petroleum

148   ether. The air dried extracted sample was transferred to 1L conical flask. Whole boiling dilute

149   sulphuric acid (1.25% w/v) was transferred to the flask containing sample and immediately

150   connected with a water cooled reflux condenser and heat it so that the contents start boiling in 1

151   min. Few drops of octanol were added to avoid bumping and risk of foaming. The flask was

152   continuously shaken and boiled for 30min. Flask is then removed and filtered through fine linen

153   (18 threads to a cm) placed in a funnel and washed with boiling water until no longer acid to litmus

154   is achieved. The residue on linen was then washed with the 200mL of boiling NaOH (1.25% w/v).

155   Immediately the flask was connected to the reflux condenser and boiled for 30min. The residue

156   was then filtered through filtering cloth and residues were thoroughly washed with boiling water.

157   The residues were transferred to Gooch crucible having thin compact layer of ignited asbestos.

158   Finally wash the residues with 15mL ethyl alcohol. The Gooch crucible was dried in air oven at

159   105±2ºC in an air oven until constant weight is achieved. The crucible was weighed and placed in

160   a muffle furnace to burnt all carbonaceous matter. Crucible was then cooled and weighed again.

161   Crude fiber was then calculated by the following formula; $crude\ fiber\%\ by\ wt = \frac{(W_1 - W_2) \times 100}{W}$,

162   where W_1 is weight of Gooch crucible before ashing, W_2 is weight of Gooch Crucible containing

163   asbestos and ash and W is weight of the dried material taken for the test. The fat content of wheat

164   was determined using the method of Association of Official Analytical chemists (AOAC, 2000)

165   with petroleum ether as solvent. In this method the fat was extracted by petroleum ether. Sample

166   of about 3.0g was weighed and added in a labelled thimble. About 150mL of petroleum ether was

167   then added to it and boiled upto 40-60ºC in 250mL boiling flask. The thimble was tightly plugged

168   with cotton wool and Soxhlet apparatus was assembled to allow it to reflux for 24h the thimble

169   was carefully removed and petroleum ether from the top was drained into another container for

170   further use. The petroleum ether was then evaporated completely in a hot air oven and desiccator

171   was weighed following cooling. The weight was determined by formula; $fat\ content\ \%\ by\ wt =$

172    $\frac{W_1 \times 100}{W}$, where W_1 is the weight of the residue in desiccator and W is initial weight of the sample.

173   Since the bulk density (BD; ρ) varies with MC, therefore, it was determined following procedure

174   described in ASABE standard S358.2 (Theerarattananoon et al. 2011). During this procedure, 100

175   g of sample was dried in a forced air convection oven (IM-115, Germany) at 103 °C for 24h. The

176   sample was then weighed on digital balance (0.01 g precision; TE-313S-DS, Germany) and MC

177   was calculated by the equation; $MC = W_{initial} - W_{final}$. MC was also determined through FT-NIR

178   spectroscopy. Flour samples of different moisture level were utilized to develop the model for

179   calibration and multivariate analysis was conducted after gathering their spectra. Unknown

180   samples were then analyzed against the calibration curve to get the moisture contents. Crude

181   protein was determined through semi micro-Kjeldahl method (AACC adopted method 46-13;

182   American association for cereal chemistry, 1995) and auto protein analyzer (Kjeltec 2400 auto-

183   analyzer, Hillerod, Denmark). Here 1 g of flour sample was used along with keeping nitrogen to

184   protein conversion factor of 5.7. AACC method 38-12 was utilized to determine the gluten

185   contents of the selected flour samples (25 g each).

Analytical method

187   NIR Omega G Analyser (Bruins Instruments, USA) was employed to analyze different parameters

188   (protein, starch, fat, moisture, gluten) of the flour and grain samples. The spectral transmissions

189   range was 700-1100 nm with 5 nm scan increment, measured at controlled room conditions of

190   24±1°C, RH 34±2%.

MIR spectroscopy

192   The FTIR transmission spectra were recorded at Burker-TENSOR 37 FTIR spectrometer with

193   Michelson interferometer. Working range of the spectrometer was 4000-12000 cm^{-1} and spectra

194   generated were interpreted on the basis overtones of different functional groups in the product.

195   Resolution of spectrometer was 4 cm^{-1}(max scan interval value was 2 cm^{-1}) with maximum scan

196   time kept at 5 sec. MIR spectra were recorded at Opus 6.0 Burker Software using Attenuated Total

197   Reflectance (ATR) unit. The reference spectrum (empty sample bottle) was utilized as background

198   measurement before loading in sample's spectra. About 8-10 g of sample was added in the sample

199   bottle to generate the spectra in diffused reflectance mode. Three spectra per sample were recorded

200   by rotating the sample bottle at 120°. The measurements were carried out under the controlled

201   conditions of temperature 24±1°C, RH 34±2%. Each spectra were the average of 3 scans per

202   object.

203 ## BP and BA quantification

204 ### Sample preparation and bleaching reaction

205 BPO and BA were quantified in wheat flour as described by the method of Abe-Onishi et al., 2004.
206 Whole wheat sample was taken and divided into two parts; one sample was kept as control and
207 BPO was added in the other. About 50g of flour was blended with 1.5mg of BPO (bleaching agent)
208 to achieve a concentration of 30µg/g. The mixture was passed through polyester sieve (400
209 mesh/cm^2) to attain the homogeneous blend and kept in dark. The bleaching reaction (performed
210 at room temperature) was monitored after every hour. The product of reaction was extracted every
211 hour for a period of 8h, finally a sample was taken at 12h and 16h. Then samples were analysed
212 by HPLC and compared with the commercial standards and calibration graph for the
213 quantification. Associated peaks (matching with the standard) were considered for the generation
214 of results.

215 ## Calibration Graph for BPO

216 Standard stock solution (SS) was generated by dissolving pure BPO (60mg/L) in diethyl ether
217 (100% purity). Working standards were obtained by diluting SS with appropriate volume of diethyl
218 ether. For BA stock solution, pure compound (100mg) was dissolved in 100mL of methanol and
219 working standards were prepared by diluting the stock solution. Calibration curve was then
220 generated by plotting the absorbance value against concentration.

221 ## Extraction Procedure for BPO and BA.

222 The standard procedure was carried out (at room temperature) in a flask with grinding stopper.
223 About 100mL of diethyl ether was added to 50g of flour (both control and the one which is already
224 mixed and sifted with BPO). This mixture was shaken vigorously on a magnetic stirrer for 10
225 minutes and left to settle for 15 minutes. Upper layer of this solution (containing the products of
226 reaction) was withdrawn through the pipette and transferred into Falcon polypropylene tube
227 (10mL) and held into ice until HPLC analysis.

228 ## HPLC method

229 The supernatant was analyzed by Waters 600 HPLC system at Inertsil ODS-80A column (5 µm,
230 4.6 x 250 mm; GL science Tokoyo, Japan) equipped Inertsil ODS-3 guard column (10mm×4mm

231   i.d.) and Waters 2996 Photodiode array detector. The detection wavelength was kept at 235nm and

232   column oven at 40°C. For isocratic separation the conditions were as follows: Water (Solvent A),

233   acetonitrile (CAN; solvent B) and Benzoic acid (Solvent C); 55% B:45%A as mobile phase for

234   1mL/min. The gradient conditions for analysis were as follows: Water-glacial acetic acid (1000:1)

235   (Solvent A), ACN-glacial acetic acid (1000:1) (solvent B); 18% B (10 min) was increased to 60%

236   B (11-15 min hold) at flow rate 1.2 mL/min and column temperature 35°C.

237   Estimation of dietary intake

238   The mean dietary intake for both BP and BA were estimated to determine the exposure rate. For

239   this purpose, 200 subjects (35-40 years of age group; random sampling) were evaluated for their

240   preferences of WF and SF as well as amount of daily intake through a questionnaire survey. The

241   SF brands, which were reported to be consumed, were actually evaluated for the presence of BA

242   in the current study. Further calculations were accomplished through the following equation:

243   $$Y = \sum(X_v \times C_v)/B_w$$

244   Where; X_v=average daily amount (kg) of wheat flour consumed by a subject

245   C_v = concentration of BA (mg/kg) as determined through HPLC in particular wheat flour sample

246   B_w = Average body weight of the subject

247   Statistical analysis

248   All samples were analyzed in triplicate (both biological and experimental replicates), therefore

249   standard error of mean (SEM) was applied using the Statistixl 1.9 Add-in package within Excel

250   2007. Two-way analysis of variance (ANOVA) was conducted, followed by post-hoc Tukey tests

251   that separated the treatment into groups (at $P < 0.01$ and $P < 0.05$). The aim was to give the

252   significant difference in the data sets from different flour samples which was not achievable

253   through univariate or one-way ANOVA.

Results

255   Both the commercially available soft flour (SF) and whole wheat flour (WF) were compared for

256   their functional properties, emulsifying properties, foaming capacity, gelation capacity, proximate

257   composition and benzoyl peroxide composition. Finally, the exposure of benzoyl peroxide was

258   compared with the daily intake capacity to observe the exposure of consumers when they are

259   consuming SF or WF. The samples of SF were collected from the flour mills which were preferably

260   consumed by the subjects. WF samples were collected from same seed variety (Inqulab 19) grown

261   under similar conditions as wheat of SF. Detailed results are as under.

262   Functional Properties

263   The oil absorption capacity (OAC; Table 1) showed that the SF sample has highest lipophilic

264   tendency of about 2.87 mL/g. Highest OAC (188 mL/100g compared to 146 mL/100g for WF)

265   and WAC (408g/100mL compared to 140 g/100mL for WF) were obtained for SF4. All considered

266   flour mill samples had almost similar results for OAC. Water Absorption Capacity (WAC) was,

267   however, higher (140 g/100mL) for WF compared to SF (<123 g/100mL).

268   The results revealed that emulsifying Activity (EA) was higher for WF (43.7%), whereas, stability

269   (ES) is higher for all SF samples (<42%). EA and ES of the WF and SF may also vary due to the

270   milling process. The emulsifying properties varied inversely, which means that WF had highest

271   EA and lowest ES. Foaming Capacity (FC) and Foaming stability (FS) collectively form the

272   foaming properties of any flour. Both of these properties are directly proportional to one another,

273   which were observed to be higher for WF (12.9% FC and 1.94% FS). FC and FS of WF is more

274   (<12% and <1% respectively) compared to all SF samples (>9% and >1% respectively). A highly

275   significant difference ($P < 0.01$) was observed when values were compared statistically with those

276   of WF.

277   Gelation capacity

278   Gelation capacity (including gelatinization temperature GnT and least Gelatinization

279   concentration LGC) are attributed and controlled by the balance between hydrophilic interactions

280   and repulsive electrostatic interactions between the water/oil and proteins (Casanova et al. 2008).

281   Results (Table 1) indicated that WF had higher gelation capacity (GnT=59.21°C; LGC=8%)

282   compared to all SF samples ($P<0.001$ when datasets were compared with the WF dataset). It can

283   also be observed that both considered parameters for gelation capacity are directly related to each

284   other such that increase in one also showed increase in the other.

285   Proximate composition

286   The proximate composition (moisture, crude fiber, fat, ash, starch, damaged starch) of all the flour

287   samples is as summarized in the Table 2. Moisture content of SF is less (3.84-4.25%), protein

288   contents of WF were higher (8.9% compared to 4.6% for SF) and total starch was also high for

289   WF (76.92% compared to 50.21% for SF). The results indicated that intense milling processes

290   have detrimental effect on several properties of the flour. Most of the components such as crude

291   protein, gluten, damaged starch datasets for SF showed highly significant difference ($P<0.01$)

292   when compared with the WF dataset. This showed that quality of flour was deteriorated while

293   processing and refining.

Benzoyl peroxide concentration

295   BPO and BA were determined simultaneously using gradient analysis (Table 3). The retention

296   times were observed to be 17.5 min for BP and 7.8 min for BA (Fig. 1 & 2). The maximum

297   absorption of BPO was obtained at 195nm and 235 nm, however, a wavelength of 235 nm was

298   kept as standard for measuring the BP in the samples, considering the possible interference with

299   the food ingredients appearing at 195nm. The calibration curve had excellent linearity for the range

300   of 0.05-16 µg/g for BPO and 0.2-15 µg/g for BA. The acquisition following isocratic gradient with

301   55% ACN, showed an excellent linearity for BP, however BA was not detected. Therefore,

302   measurements at gradient conditions were kept as standard for the analysis.

303   BPO is a free radical initiator and it causes the oxidation of carotenoids by free radical mechanism.

304   The process (Fig. 3) leads to the formation of benzoic acid (BA) as a by-product (Saiz et al. 2001;

305   Shan et al. 2007; Sumnu & Sahin 2008). No BA or BP were observed for the Whole Wheat Flour

306   sample (WF) (Fig. 2). In the controlled samples, 30 µg/g of BP was added to the wheat flour

307   samples (grown under standard field conditions) and 99.5% recovery (29.5 µg/g) was observed

308   soon after adding. After 3h of bleaching, the amount of BP reduced significantly to 4 µg/g (Fig. 4)

309   that reached to zero after 8h of exposure. The contents of BA were observed to be 2.84 µg/g as

310   recovered soon after the addition. This quantity increased to 8.9 µg/g after 3h and increased further

311   to 13.5 µg/g after 8h of exposure. The contents of BA were determined again after 12h though

312   very small increase in quantity was observed (13.75 µg/g), which shows that the process of

313   conversion got stabilized with slight variation. To confirm this, the flour was analyzed again after

314   16 h and quantity of BA didn't change much (13.77 µg/g). Local standards for maximum

315   acceptable quantity have not yet been specified however, international standards (60 µg/g for BP;

316   0-5 mg/kg for BA as per body weight by JECFA acceptable daily intakes) were considered in the

317   current study. Analysis of WF and SF available commercially for BP and BA showed higher rates

318   of BA in the SF samples (Table 4). This indicated higher amount of BA intake when SF based

319   products are consumed.

320   ## Estimation of daily intake

321   Estimation of dietary exposure of BA through just wheat flour consumption was estimated (Table

322   5). Since flour is important parameter of people diet, therefore, it was deemed necessary to give

323   the level of exposure through flour only. Rest of the food groups such as noodles and drinks,

324   though unavoidable, also contain certain amount of BA but they were not considered in the current

325   study. As evaluated through questionnaire study most of the considered subjects (72.5) were

326   consuming SF purchased from the flour mills.

327   HPLC was used for quantification of BP in both white and whole wheat flour samples. BA was

328   also determined because BP was decomposed into benzoic acid within limited days (Fig. 4). A

329   research on bleaching agents including BPO and BA by HPLC during bleaching process of wheat

330   flour. The retention time of BP was 17.5 min and that of BA was 7.6 min. After 30h of bleaching

331   BPO concentration was 11 ppm. After 3 months its concentration was reduced to 6 ppm. These

332   results demonstrated that when benzoyl peroxide added to flour their greater amount was

333   decomposed into benzoic acid within limited days of treatment. The analytical results of present

334   study showed that that retention time of BPO was 17.5 minutes and BA was 7.5 minutes at 235nm.

335   In WF samples no content of BPO and BA were found. In white flour samples BP content ranges

336   from 6.6-21 mg/kg and BA content ranges from 13-28 mg/kg.

337   # Discussion:

338   Whole wheat flour (WF) and Soft Flour (SF) were compared for their properties such as functional

339   properties, emulsifying properties, foaming capacity, gelation capacity, proximate composition

340   and benzoyl peroxide composition. The comparison was developed to aware and understand the

341   choice of flour in daily meals and their potential associated risks.

342   The oil absorption capacity (OAC) of the flour varies with the intrinsic properties such as amino

343   acid composition, protein conformation, hydrophilic-hydrophobic balance of amino acids, steric

344   factors as well as lipid and carbohydrate composition of a flour sample (Mao & Hua 2012). Results

345   showed that SF samples had more non-polar side chains compared to WF which enhanced the oil

346   binding capacity of SF and reduced its water binding capacity. Higher OAC (188% compared to

347   146% for WF; Table 1) represents that the flour can retain flavor and could have optimum uses in
348   different food products such as bakery items. More hydrophobic sites as in case of SF (OAC
349   >175%), as represented through OAC value, are important for bakery items in which fat absorption
350   is desirable (Chandra et al. 2015; Chassagne-Berces et al. 2011). Water Absorption Capacity
351   (WAC) is important since it gives the capacity of a flour to have higher hydration capacity, lower
352   WAC (<123%) in case of SF and higher in case of WF (140%) means that excessive grinding in
353   the flour mills and sieving has resulted in the modification of protein structure. Another factor of
354   higher WAC could be the fibers retaining the water. Reduction of theses fibers in case of SF has
355   reduced its capacity to absorb water (Onipe et al. 2017; Shewry 2009). WAC is an important
356   component since it allows the food to have sufficient water retention and transfer of this water
357   upon consumption. The interaction of protein with water is usually determined through water
358   hydration, holding, water retention and water imbibing. WAC favors another phenomenon called
359   Water Hydration Capacity (WHC). WHC is a physical feature and describes the ability of flour
360   structure to prevent water from being released from a protein structure. During food processing
361   the protein structures are deteriorated which may influence the WHC of wheat as in case of SF.
362   Swelling, another important functional property referred to as spontaneous uptake of water by
363   protein matrix, is indirectly related to WHC. Since SF is lower in WHC therefore its swelling
364   capacity is more (>18 compared to 17.8 in case of WF), which ensures longer life of bakery items.
365   Water retention is very important for protein functionality which determines the cationic, non-
366   ionic and anionic polar sites of the protein molecules on the flour surface (Zayas 1997). Lower
367   WHC means that proteins have less water binding capacity or they are "salted out" that takes place
368   when the proteins are precipitated out. (Ramaswamy et al. 2013) showed that the higher water
369   retention is associated with linear form of arabinan compared to its branched form. The branched
370   form of arabinan is unhealthy for consumption, therefore WF should be the choice for consumption
371   instead of SF. The wheat kernel is usually composed of 12-16% protein, during milling process
372   the protein content and wheat gluten are separated, though gluten is insoluble content of protein.
373   The wheat gluten is further damaged, which may be the reason that in the current results (Table 2)
374   gluten was observed to be higher in concentration compared to protein, since the milling and other
375   processes separated it out.
376   (Ali et al. 2014) reported that lesser water retention properties are directly related to the damaged
377   starch contents of the wheat. During milling process, the flour obtained comes from the endosperm

378   which is rich in starch contents. The physical process of grinding during which the cylinders move

379   closer and closer causes the starch granules to rupture. This results in the damaged starch contents

380   and also has detrimental effect on the protein composition. The higher water retention reflects the

381   absorption of water in the starch granules, which also limit its propagation. In case of WF, the

382   flour is produced through grinding the whole seed (endosperm and periphery) which is rich in

383   pentosan, starch, ash and protein in more or less original form.

384   The emulsifying properties vary inversely with the insoluble protein fractions and polysaccharides

385   (Chove et al. 2001; Haruna et al. 2011). The unfolding of proteins when at interface water/oil

386   determines the Emulsifying Stability (ES) and Emulsifying Activity (EA). ES of protein is related

387   to the ability to minimize the interfacial tension between oil and water when in emulsion. Surface

388   activity is determined through the ability of protein to migrate, deploy, absorb as well as rearrange

389   at interface. The region of the minimum solubility of proteins (isoelectric region) was the region

390   of least soluble and minimum emulsifying capacity. The emulsifying properties vary with the two

391   effects 1) absorption of the protein at oil or water interface results in a substantial decrease in

392   interfacial energy 2) structural, electrostatic and mechanical energy barriers are caused by the

393   interfacial layer that opposes destabilization (Chaparro Acuña et al. 2012; Kumar et al. 2011). This

394   property makes both EA and ES opposite to each other. Higher EA of WF (>40%) makes it a

395   preferable choice compared to SF.

396   Akintayo et al. (1999) showed that Foaming Capacity (FC) is associated with the flexibility of

397   protein molecules which reduces the surface tension as well as the globular protein which can

398   hinder surface denaturation, therefore, leading to a low FC (<9% in case of SF). The foaming

399   capacity indicates that proteins have active sites on the flour. Soluble proteins reduce the surface

400   tension when at interface between the fluid surrounding the molecules and air bubbles, which

401   blocks the coalescence. Protein molecules can also be deployed, which interact with each other to

402   give multilayer or film protein which increases the flexibility of air-liquid interface. This results

403   in harder foam due to unbreakable bubbles (Adebowale & Lawal 2003). Higher FC and Foaming

404   stability (FS) of WF indicates that protein structures are not denatured yet and they still carry the

405   capacity; however, in case of SF they seem to have lost their arrangement due to which reduced

406   foaming properties has been observed.

407   Protein gelation is very important in several vegetables and other food items. The effective

408   overlapping of the functional groups between adjacent protein moieties is very important for the

409   gel network formation. Higher gelation capacity (Gelatinization temperature) for WF indicates that

410   this may not be a good choice in bakery items.

411   The flour at the beginning of the process comes from the endosperm, which is rich in starch and

412   as it reaches the end of grinding, flour comes from periphery which is rich in ash, pentosan and

413   protein. During the milling process, the seeds pass through heavy grinders to attain fine powdered

414   flour. This flour is further sieved and final product, obtained after series of sieving and treatment

415   with BP, is packed and sold as commercial white flour used for bakery items. Most of the

416   components such as crude protein, gluten, damaged starch datasets showed highly significant

417   difference ($P<0.001$) with the WF dataset. This shows that quality of SF was deteriorated during

418   processing and refining.

419   Flour mills consume benzoyle peroxide (BP) to improve the appearance and white colour of flour.

420   BP is a free radical initiator and therefore, it causes the oxidation of carotenoids by free radical

421   mechanism. The process (Fig. 3) leads to the formation of benzoic acid (BA) as a by-product (Saiz

422   et al. 2001; Shan et al. 2007; Sumnu & Sahin 2008). (Onishi et al. 2004a) reported that Chigasaki

423   Health Centre in Kanagawa Prefecture in Japan observed about 60-100 µg/g of BA in December

424   1999, which was found to be due to BP introduced in the food items; therefore BP was being

425   decomposed into BA. Current study suggested that not all BP was converted into BA, therefore

426   traces are still left in the sample which was in compliance with the findings of the previous studies

427   (Onishi et al. 2004a; Ponhong et al. 2015). Further analyses of SF indicated that all samples had

428   traces of BP and excessive amount of BA in them. Study by (Ponhong et al. 2015) indicated that

429   not all BP could get converted to BA and slight amount of BA is also introduced during the

430   bleaching process that helps in the initiation of conversion process. The Joint FAO/WHO Expert

431   Committee on Food Additives (JECFA) has approved the quantity of BP allowable to be about 0-

432   40 mg/kg which has also been approved by (Organization 2001) considering the requirement of

433   whitening of flour. However, increased temperature during baking also speeds up the processes of

434   metabolism of BP to it's by products (and BA in particular). The maximum allowable level for

435   BA is 150 mg/L according to European community food safety regulations (EC, 1995), which

436   means daily intake of 55.8 mg of BA per person or 0.8 mg/kg body weight assuming 70 kg of

437   weight is allowed (Solomon et al. 2005). According to JECFA acceptable daily intake was 0-5

438   mg/kg for BA and benzoate. Intake of benzoic acid in sensitive persons, lower than 5 mg/kg of

439   body weight per day has been observed to cause non-immunological contact reaction. A few

440 studies have reported strong allergic reactions such as urticarial, pruritus and rhinitis to benzoic

441 acid and benzoate exposure. According to current study, Table 5, SF consumers gain 44.3

442 mg/kg/bw of BA per day which is above maximum allowable intake (5 mg/kg/bw) compared to

443 those who consume WF.

444 Wheat flour is an unavoidable commodity. This shows that white SF as produced out of milling

445 process are contributors of excessive amount of toxic benzoic acid (BA) in the consumers' body.

446 Results of current study indicated very high amount of BA entering in human body upon

447 consumption of SF. In China standard limit of BPO is not exceed 60 mg/Kg; the maximum content

448 of BP in wheat flour is 80 mg/Kg in the US, in Japan 300 mg/Kg, and also 50 mg/Kg in United

449 Kingdom. In China, permissible amount of BPO are 0.045, 0.05 0.06 g/kg according to standards

450 of food additives regulation (Wei et al. 2006). According to Japanese regulations allow the use of

451 diluted BPO (19-22% w/w) in wheat flour which is lesser than 0.30 g/kg. In France, the use of BP

452 is strictly banned. In UK and USA the permitted level BPO are 0.05 g/kg, 0.045 g/kg respectively

453 (Saiz et al. 2001). These standard limits demonstrated that the concentration of BP in SF was

454 within permissible limit, however when this value was calculated on the basis of Avg. Daily Intake

455 (ADI) and Estimated Daily Intake (EDI) (Table 5) it was observed that influx of BA in the body

456 of consumers is very high, which is 44.3 mg/kg/day compared to allowable level of 0-5 mg/kg/day.

457 Current study showed an EDI of 78.3 g/day if a person consumes Results revealed that regular

458 consumption of SF has profound effect on human health, therefore WF should be a preferable

459 choice instead.

460 ## Conclusions

461 Wheat flour is one of the most important ingredients of food being consumed most frequently. To

462 improve the baking quality, SF is often used instead of WF. Increased demands of fine texture and

463 bleached colour has led flour millers to add enhanced concentration of BPO and extensive milling

464 along with sieving. Extensive milling and increased BPO reduce the nutritive value of SF and

465 enrich it with the toxicant such as BA (as degradation product of BPO). Among various parameters

466 of flour quality, protein and ash content in WF sample was more than SF samples. Therefore, there

467 is need to improve wheat flour quality being sold in the market by limiting the rate of BPO added

468 as bleaching agent as well milling process should be improved. HPLC analyses effectively

469 demonstrated the dissociation of BPO to BA, which means that BA in SF was due to BPO added

470 as bleaching agent.

471

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Figure 1

HPLC chromatogram of BPO (17.56 min) and BA (7.018 min) in WF when induced with 30μg/g of BP.

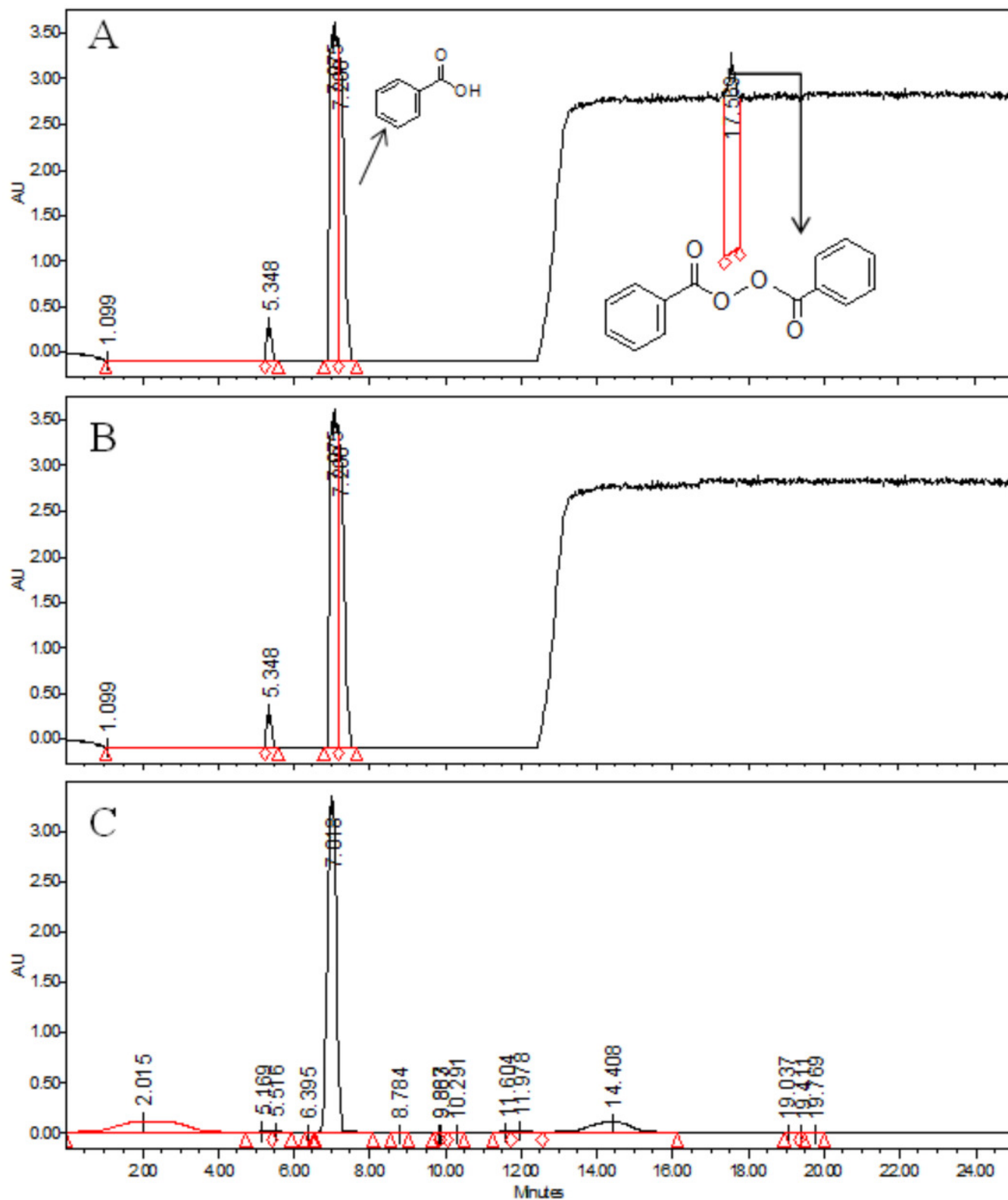



Figure 2

HPLC chromatograms for BPO and BA

HPLC chromatograms for BPO and BA in A) commercial soft flour (SF1) B) whole flour (WF) without any addition of preservatives.

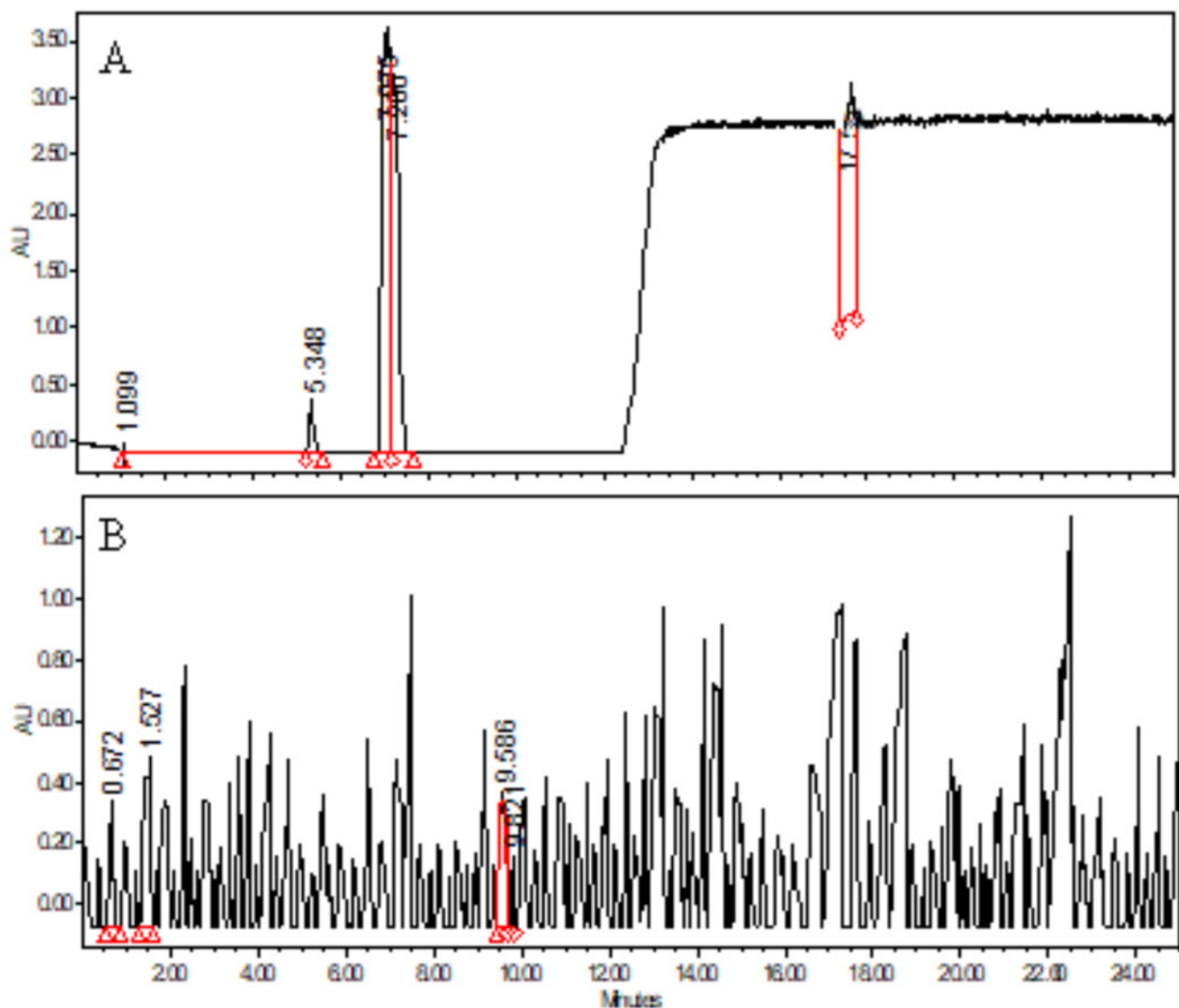



Figure 3

Possible pathway for the degradation of benzoyl peroxide (BPO) to benzoic acid (BA) as observed through current research.

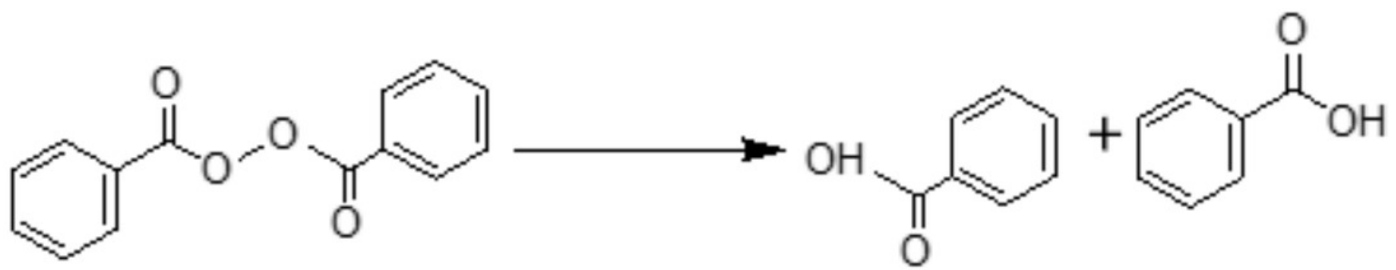
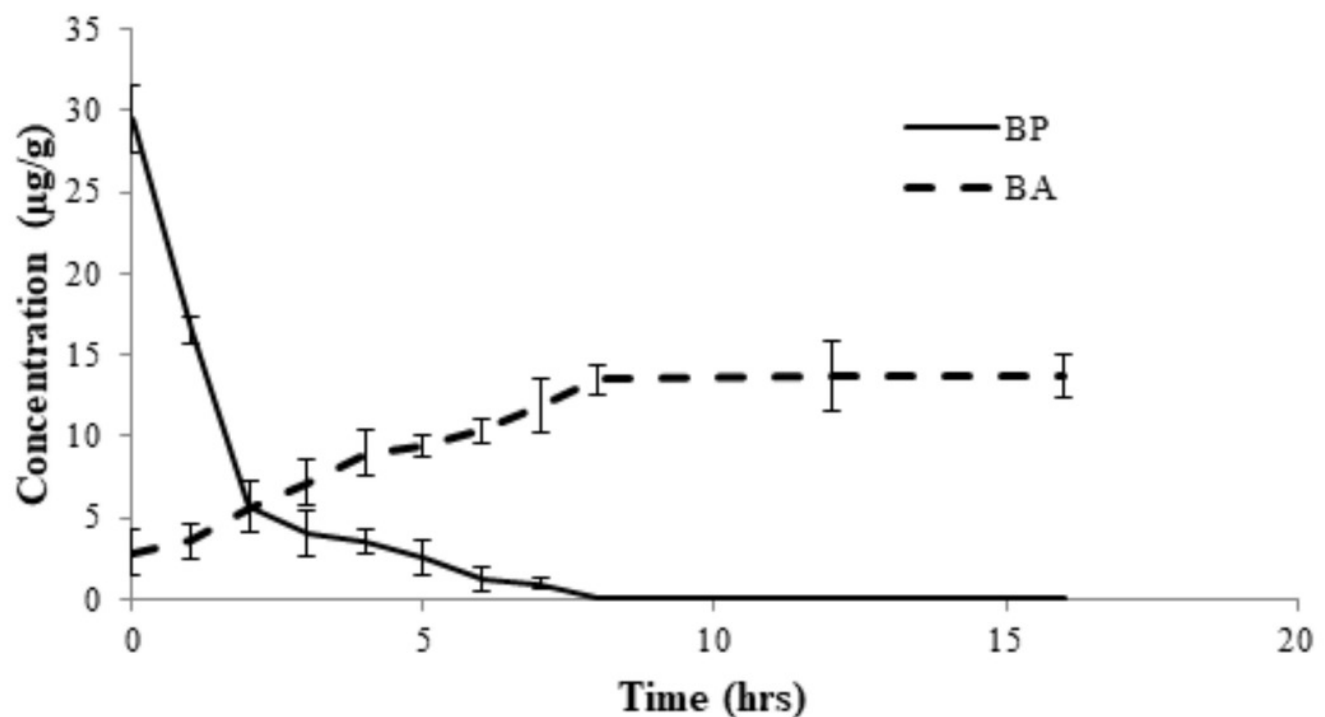



Figure 4

Rate of conversion of benzoyl peroxide (BP) to benzoic acid (BA) in flour at varied time intervals.

Rate of conversion of benzoyl peroxide (BP) to benzoic acid (BA) in flour at varied time intervals. About 50g of flour was amended with 30µg/g of BPO as bleaching agent. Rate of dissociation was observed over a period of 16h.

Functional Properties of the whole wheat flour (WF) and soft flour (SF) samples (*P<0.05; **P<0.01; significant difference as calculated through ANOVA).

Notes: *SC=swelling capacity; WAC=water absorption capacity; OAC=oil absorption capacity; EA=emulsifying activity; ES=emulsifying stability; FC=foaming capacity; FS=foaming stability; GnT=gelatinization temp.; LGC=least gelatinization concentration; BD=bulk density

Samples	SC at 95°C (g/mL) Mean±SD	WAC (g/100mL) Mean±SD	OAC (g/100g) Mean±SD	EA (%) Mean±SD	ES (%) Mean±SD	FC (%) Mean±SD	FS (%) Mean±SD	GnT (°C) Mean±SD	LGC (%) Mean±SD	BD (ρ) Mean±SD
WF	17.8±1.2	140±5.77	146±9.33	43.7±2.61	38.4±5.77	12.9±1.02	1.94±0.07	59.21±1.39	8±1.65	0.76±0.048
SF1	19.4±0.98	121±4.04	181±8.75	37.84±3.47	44.6±7.54	8.97±1.22**	0.77±0.065	41.21±1.66*	6±0.98*	0.23±0.06
SF2	18.8±0.97	123±8.93	175±7.63	36.44±7.12	45.4±8.75	8.66±1.08**	0.84±0.078	38.7±1.86*	4±0.87**	0.24±0.056
SF3	19.6±0.81	120±2.36	184±8.55	38.45±3.22	43.5±8.41	8.71±1.76**	0.76±0.065	37.74±2.06*	4±0.65**	0.21±0.081
SF4	19.7±1.72	118±3.45	188±9.34	36.7±4.21	42.9±7.66	8.34±0.51**	0.74±0.045	38.45±2.76*	4±0.77**	0.26±0.056

Proximate composition of whole wheat flour (WF) and soft flour (SF) samples (*$P<0.05$; **$P<0.01$; significant difference as calculated through ANOVA).

Samples	Moisture (%) Mean±SD	Ash (%) Mean ±SD	Crude fibre (%) Mean±SD	Fat (%) Mean ±SD	Crude Protein (%) Mean±SD	Gluten (%) Mean± SD	Starch (%) Mean±SD	Sugar (%) Mean± SD	Damaged starch (%)Mean ±SD	Pentosan (%) Mean±SD
WF	8.64±0.054	1.6±0.08	1.44±0.076	2.29±0.15	8.9±1.24	14.4±0.78	76.92±4.32	16.92±1.07	45.84±5.42	1.6±0.07
SF1	4.25±1.23*	0.54±0.064	0.34±0.076*	1.54±0.98	4.65±0.86**	7±1.23**	50.26±1.86**	23.41±3.74	88.36±4.32**	0.25±0.054
SF2	4.22±0.83*	0.55±0.043	0.34±0.07*	1.32±0.05	4.32±0.85**	7.5±2.21**	50.4±2.77**	23.15±8.75	87.22±2.05**	0.23±0.055
SF3	3.84±0.67**	0.45±0.081	0.32±0.05*	1.12±0.063	4.15±1.65**	6±1.22**	50.21±1.24**	23.4±6.34	82.3±1.24**	0.24±0.07
SF4	3.9±0.17**	0.34±0.049	0.33±0.074*	1.12±0.048	4.12±0.08**	6.5±1.07**	50.21±4.32**	23.25±3.45	85.62±2.23**	0.26±0.05

Recoveries of Benzoyl peroxide (BP) and Benzoic acid (BA) from the flour.

Components	Added amount (µg/g)	Recovery (%)	
		Gradient Mean±SD	Isocratic Mean±SD
Benzoyl peroxide	7	$98.1_{\pm0.55}$	$99.2_{\pm0.41}$
	30	$99.5_{\pm0.61}$	$99.3_{\pm0.55}$
	60	$96_{\pm0.25}$	$99.3_{\pm0.21}$
Benzoic acid	5	$91.3_{\pm0.44}$	-
	10	$91.3_{\pm0.31}$	-

Concentrations of BP and BA in different flour samples.

WF=Whole wheat flour; SF= Soft flour

Samples	Contents (µg/g)	
	BP Mean±SD	BA Mean±SD
WF	0	0
SF1	$2.45_{\pm1.1}$	$68.11_{\pm14.1}$
SF2	$3.41_{\pm1.02}$	$71.4_{\pm12.31}$
SF3	$2.54_{\pm0.94}$	$71.51_{\pm15.84}$
SF4	$5.77_{\pm0.33}$	$72.55_{\pm15.33}$

Estimated intake of BA on the basis of intake of flour by consumers.

Allowable EDI for BA=0-5 mg/kg/bw

Food group	Consumer (%)	Avg. Daily intake of flour (g/day) Mean±SD	Estimated daily intake of BA (mg/kg/bw) Mean±SD
SF	72.5	$78.3_{\pm2.3}$	$44.3_{\pm1.36}$
WF	10.5	$72.1_{\pm3.3}$	$1.34_{\pm1.45}$
Alternate	17	$68.3_{\pm1.23}$	-