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## Omega-3 enriched biscuits with low levels of heat-induced toxicants: effect of formulation and baking conditions

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<b>Abstract:</b>	<p>Unconventional formulation and baking conditions were exploited for obtaining omega-3 polyunsaturated fatty acids enriched biscuits. A monoglyceride-flaxseed oil-water gel was used to obtain biscuits which had physical and chemical properties analogous to those of a control sample prepared with palm oil. To reduce fat oxidation and acrylamide and furan formation, the dough was baked at different temperature, time and pressure (i.e. varying from 101.33 to 0.15 kPa) conditions according to a central composite design. Baking at high temperature and reduced pressure allowed to obtain biscuits with acceptable water content and colour, while minimizing omega-3 fatty acids oxidation and acrylamide and furan formation. The biscuits best responding to these characteristics were obtained by applying the combination 174 °C-3.99 kPa-45 min. The low pressure generated inside the oven likely exerted a stripping effect towards acrylamide and furan as well as oxygen thus preventing toxicants to accumulate and lipid oxidation to occur. This study highlighted that the use of monoglyceride-flaxseed oil-water gel combined with baking under reduced pressure is potentially applicable at the industrial level to obtain nutritionally enhanced biscuits, while simultaneously preventing the occurrence of degradation reactions and toxic molecules formation. Due to the worldwide diffusion of cereal-based foods, including sweet biscuits, this formulation and process strategy could have a great economic impact.</p>

1 **Omega-3 enriched biscuits with low levels of heat-induced toxicants: effect of formulation and baking conditions**

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9 **Abstract**

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11 enriched biscuits. A monoglyceride-flaxseed oil-water gel was used to obtain biscuits which had physical and chemical  
12 properties analogous to those of a control sample prepared with palm oil. To reduce fat oxidation and acrylamide and  
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21 occurrence of degradation reactions and toxic molecules formation. Due to the worldwide diffusion of cereal-based  
22 foods, including sweet biscuits, this formulation and process strategy could have a great economic impact.

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24 **Keywords:** omega-3 polyunsaturated fatty acids, oxidative stability, acrylamide, furan, monoglyceride-oil-water gel,  
25 low pressure baking

## 28 Introduction

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29 The health benefits associated with the consumption of omega-3 polyunsaturated fatty acids (PUFAs) are well  
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40 documented and include the reduction of the occurrence of certain chronic diseases as well as the improvement of brain,  
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61 retina and nervous system functions (Huang et al. 2004; Salem and Eggersdorfer 2015; Guichardant et al. 2015; Tani et  
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82 al. 2015; Koh et al. 2015). The most important omega-3 PUFAs of our diet are  $\alpha$ -linolenic acid, eicosapentaenoic acid  
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103 and docosahexaenoic acid. Fish, fish oils, some vegetable oils, and microalgae are sources of omega-3 fatty acids (Xie  
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124 et al., 2015). However, current dietary habits, especially in Western countries, have considerably decreased the daily  
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145 intake of these foods and thus omega-3 PUFAs below the recommended dose (Salem and Eggersdorfer 2015; Ferguson  
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166 et al. 2014; Ferguson et al. 2010). The development of functional foods enriched with omega-3 PUFAs can help to  
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187 promote a correct dietary style by delivering appropriate amounts of these nutrients, while meeting consumer  
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208 preferences and habits. Bakery products, such as sweet biscuits, could be good candidates for such fortification since an  
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229 improvement of their healthy properties would have great impact on a large portion of population due to their high  
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240 frequency of consumption. In the effort to develop omega-3 PUFAs fortified foods, one of the most frequently applied  
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261 strategy is the addition in the formulation of oil naturally containing omega-3 fatty acids (i.e. cod liver oil, flax-seed oil,  
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282 canola oil) (Santhanam et al. 2015), although other omega-3 fatty acids sources can be also used (e.g. microalgal  
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303 biomasses) (Gouveia et al. 2008).  
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324 Different issues have to be overcome in the attempt to develop bakery products incorporating omega-3 rich oils. A  
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345 major issue is represented by omega-3 PUFAs susceptibility to oxidation (Kamal-Eldin and Yanishlieva 2002). Besides  
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366 nutrient depletion and undesired changes of the food sensory properties, oxidative reactions may be responsible for the  
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387 formation of toxic compounds. For instance, the oxidation of PUFAs results in significant generation of dietary  
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408 advanced lipid oxidation end-products (ALEs), that may exert cytotoxic and genotoxic effects (Kanner 2007; Awada et  
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429 al. 2012). In addition, the substitution of plastic fats with liquid oil could greatly affect the baking performances and  
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445 thus the product quality (Brooker 1996). An emerging strategy to mimic the structure provided by plastic fats is the use  
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466 of oils structured by molecules able to generate self-assembling networks (Pernetti et al. 2007). Saturated  
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488 monoglyceride-based gels are among the most promising fat-substitutes able to bring new or improved functionality to  
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503 food products (Batte et al. 2007; Marangoni et al. 2007). Different examples of the use of monoglyceride gels as  
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524 structuring phase in bakery products have been reported in the literature, especially in the attempt to reduce the  
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545 saturated fat content (Goldstein and Seetharaman 2011; Manzocco et al. 2012a and b; Calligaris et al. 2013). Results  
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566 demonstrated that this structural approach could be a pursuable strategy along with a careful re-set up of the processing  
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588 conditions. In fact, the substitution of a lipid phase with a monoglyceride-oil-water gel, by favoring precursor's  
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603 encounter in the water phase surrounding monoglyceride lamellas, resulted in an increase of acrylamide formation  
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59 (Anese et al. 2011; Manzocco et al. 2012b). As known, high levels of the toxic and suspected carcinogenic acrylamide  
60 and furan are formed in a wide range of staple foods during heating, including cereal-based products. Average  
61 acrylamide and furan concentrations of 333 µg/kg and 18 µg/kg, respectively, are reported for these products (EFSA  
62 2011; EFSA 2012). It has been recently demonstrated that cereal-based products contribute on average about 9% to  
63 total acrylamide dietary intake (Friesling et al. 2013), and have been identified as major contributors to furan exposure  
64 in the children and adolescent population (EFSA 2011). A number of ways have been suggested to reduce the levels of  
65 acrylamide and furan in foods, as recently reviewed by Zhang and Zhang (2007), Pedreschi et al. (2014), Curtis et al.  
66 (2014) and Anese et al. (2013). We have previously demonstrated that heating under reduced pressure could allow  
67 acrylamide formation in coffee to be minimized (Anese et al., 2014).

68 The objective of the present study was to exploit formulation and baking conditions suitable for obtaining omega-3  
69 PUFAs enriched short dough biscuits. In particular, a monoglyceride-flaxseed oil-water gel was developed and  
70 characterized, and subsequently used instead of palm oil to prepare the dough. After moulding, the dough was subjected  
71 to baking under different process conditions. Temperature, time and pressure (the latter ranging from the atmospheric  
72 value to 0.15 kPa) were varied according to a three variable face centred central composite design and the effects of  
73 these variables on water content, colour and peroxide value were evaluated. Moreover, this study was designed to  
74 investigate whether baking under reduced pressure could allow acrylamide and furan formation in biscuits to be  
75 minimized.

## 76 **Materials and methods**

### 77 **Materials**

78 Myverol™ saturated monoglyceride (MG) (fatty acid composition: 1.4% C14:0, 59.8% C16:0, 38.8% C18:0; melting  
79 point 68.05 ± 0.5 °C) was kindly donated by Kerry Ingredients and Flavour (Bristol, United Kingdom).

80 Isooctane, 1-butanol and sodium bicarbonate were from Carlo Erba Reagents (Milano, Italy); methanol, ethyl ether  
81 stabilized with 2% of ethanol, 2,3,3[<sup>2</sup>H<sub>3</sub>] acrylamide (d<sub>3</sub>-acrylamide), anhydrous sodium sulphate, co-surfactant mixture  
82 of palmitic and stearic acids 1:1 (w/w) were from Sigma-Aldrich (Milano, Italy); barium chloride and ferrous sulphate  
83 were from Panreac (Barcellona, Spain); ammonium thiocyanate was from Emsure (Damstadt, Germany). Type 0 wheat  
84 flour, sucrose, eggs, skimmed milk powder, table salt, sodium bicarbonate, baking powder, palm oil were purchased in  
85 a local market. Flaxseed oil was from Solimè (Cavriago, Italy). According to producer indications, total omega-3 fatty  
86 acids in flaxseed oil were 53.4%.

90 Preparation of the monoglyceride–flaxseed oil–water gel

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The monoglyceride–flaxseed oil–water gel, hereafter called hydrogel was prepared according to the slightly modified method of Calligaris et al. (2010). Myverol™ saturated monoglyceride (MG) was mixed with a co-surfactant mixture of palmitic and stearic acids 1:1 (w/w) in a ratio of 5:1 (w/w). The water phase consisted of 1 mM NaHCO<sub>3</sub> in deionized milli-Q water to promote the partial neutralization of the co-surfactant mixture and obtain a properly swollen phase. The hydrogel was obtained by mixing the flaxseed oil, previously heated at 70 °C, with the MG/co-surfactant mixture up to complete dissolution, followed by the addition of the heated (70 °C) water solution. The two phases were then homogenized by using a high speed homogenizer (D125, Ika-Werke, Staufen, Germany) at 59,000 × g for 90 s. Afterwards, the mixture was cooled at 4 °C in an ice bath and then stored at 4 °C for 24 h before use. The concentration by weight of each constituent in the monoglyceride–flaxseed oil–water gel was as follows: MG/co-surfactant, 4.8%, flaxseed oil, 47.6%, water solution, 47.6%.

Short dough biscuits preparation

The non-water ingredients consisted of wheat flour, sucrose, eggs, skimmed milk powder, table salt, baking powder, palm oil or MG-hydrogel and were added to the recipe at 54, 15, 0.7, 0.3, 0.2, 1.1, and 29% on total weight, respectively. The fat concentration of the hydrogel added to the formulation corresponded to 15%. All the ingredients except the palm oil or hydrogel and the wheat flour were first mixed for 15 min by using a kneader (Hobart, N50CE, Ohio, USA). Afterwards, the palm oil or hydrogel was incorporated and mixed for 5 min, followed by the addition of the wheat flour and kneaded for further 15 min. After mixing, the dough was sheeted to 8 mm thickness and cut to a diameter of 50 mm. The samples were baked in an oven (5Pascal, VS-25 SC, Trezzano S/N, Milano, Italy) connected to a rotary vacuum pump (BOC Edwards, E2M40, Crawley, West Sussex, UK) able to achieve a pressure of 1.33 Pa in few seconds when the oven was empty. Once the desired temperature was reached, two dough biscuits, previously weighed (approximately 5 g each), were introduced in the central rear part of the oven on a plate and the vacuum pump was immediately switched on. The time needed to achieve the desired vacuum was less than 10 s. Computation of treatment duration started once the set pressure value was achieved. Baking was carried out at different pressures, temperatures and times according to a central composite design. Preliminary trials, were carried out at 180 °C and atmospheric pressure for 20 min. After the treatments, samples were immediately removed from the oven and cooled to room temperature. Afterwards they were transferred into plastic vessels with pressure lid and stored at -18 °C until analyses were performed.

121 Temperature monitoring and thermal effect computation

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123 Temperature changes during baking were measured by a copper-constantan thermocouple probe (Ellab, Denmark),  
124 whose tip (2.0 mm) was placed on the sample surface. The thermal effect  $F$  (min) was computed using the following  
125 equation (Ball, 1923):

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$$F = \int_0^t 10^{(T-T_{ref})/z} \cdot dt \quad \text{eq. 1}$$

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127 where  $T_{ref}$  is the reference temperature, which was chosen equal to 200 °C, a baking process being generally carried out  
128 at temperatures around 200 °C,  $T$  is the actual temperature of the treatment (°C),  $t$  is the time (min) of the treatment, and  
129  $z$  represents the increase in temperature that causes a 10-fold increase in the reaction rate, which was reported to be  
130 equal to 56 °C for the browning reaction (Sacchetti et al. 2009).

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133 Acrylamide analysis

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136 Acrylamide determination was carried out according to the method of Anese et al. (2009). Briefly, 1000 µL of an  
137 aqueous solution of 2,3,3-[<sup>2</sup>H<sub>3</sub>] acrylamide (d<sub>3</sub>-acrylamide) (0.20 µg/mL) as internal standard and 15 mL of water Milli  
138 Q (Millipore, Italy) were added to 1 g of finely ground biscuit weighed into a 100 mL centrifuge tube. After extraction  
139 at 60 °C for 30 min under magnetic stirring, the mixture was centrifuged at 12,000 x g for 15 min at 4 °C (Beckman,  
140 Avanti Centrifuge J-25, Palo Alto, CA, USA). Aliquots of 10 mL of the clarified aqueous extract were cleaned-up by  
141 solid phase extraction (SPE) on an Isolute Env+, 1 g (Biotage, Sweden). The volume of the eluted fraction was reduced  
142 under vacuum, to about 1.5 – 2 mL by using a rotary evaporator (Laborata 4001, Heidolph, Schwabach, Germany) at a  
143 temperature of 80 °C and filtered through a 0.45 µm membrane filter before the HPLC-MS analysis. LC-ESI-MS-MS in  
144 positive ion mode analyses were performed by a Finnigan LXQ linear trap mass spectrometer (Thermo Electron  
145 Corporation, San José, CA, USA) coupled to a Finnigan Surveyor LC Pump Plus equipped with a thermostated  
146 autosampler and a thermostated column oven. The analytical column was a Waters Spherisorb ODS2 (250 x 2.0 mm, 5  
147 µm). Elution was carried out at a flow-rate of 0.1 mL/min, in isocratic conditions at 30 °C using as mobile phase a mixture  
148 of 98.9% water, 1% methanol and 0.1% formic acid (v/v/v). Full scan MS/MS was carried out by selecting the ions at  
149  $m/z$  72 and  $m/z$  75 as precursor ions for acrylamide and d<sub>3</sub>-acrylamide respectively. The area of the chromatographic  
150 peaks of the extracted ion at  $m/z$  55, due to the transition 72 > 55, and at  $m/z$  58, due to the transition 75 > 58, were used  
151 for the quantitative analysis. The quantitative analysis was carried out with the method of the internal standard. The

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150 relative response factor of acrylamide with respect to d<sub>3</sub>-acrylamide was calculated daily by analyzing a standard  
151 solution. For each run, analyses were made at least in duplicate. Acrylamide concentration was expressed as ng/g of dry  
152 matter.

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154 Furan analysis

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156 Furan analysis was carried out by combining SPME and GC-MS analysis according to slight modifications executed on  
157 the method of Bianchi et al. (2006). SPME experiments were performed with a 85 mm carboxen-polydimethylsiloxane  
158 (CAR-PDMS) fibre (Supelco, Bellfonte, PA, USA). Aliquots of 2 g of sample were added with 2 mL NaCl 20% (w/w)  
159 water solution of d<sub>4</sub>-furan (internal standard with a concentration equal to 30 µg/kg) and were placed in 20 mL sealed  
160 vials. After 5 min incubation at 40 °C, the fibre was exposed to the vial headspace at 40 °C for 20 min, under constant  
161 magnetic stirring. Desorption was carried out at 270 °C for 2 min. Two fiber blanks were run between each sample to  
162 avoid potential “memory effects”. An Ultra-Thermo TRACE GC (Thermo Scientific, Waltham, MA, USA) equipped  
163 with a DSQ II detector (Thermo Scientific, Waltham, MA, USA) was used for GC-MS analysis. Helium was used as the  
164 carrier gas at a flow rate of 1 mL/min; the gas chromatograph was operated in splitless mode with the PTV injector  
165 maintained at 270 °C and equipped with a PTV multi-baffled liner (i.d. 1.5 mm, Thermo Scientific, Waltham, MA,  
166 USA). A Rxi-5ms (5% diphenyl 95% dimethylpolysiloxane) (30 m x 0.25 mm, 0.5 µm) capillary column (Thermo  
167 Scientific, Waltham, MA, USA) was used. The following GC oven temperature program was applied: 40 °C for 5 min,  
168 15 °C/min to 300 °C. Transfer line and source were maintained at 270 °C and 200 °C, respectively. The mass  
169 spectrometer was operated in selected-ion monitoring mode (SIM) by recording the current of the following ions: *m/z*  
170 68 and 39 for furan and *m/z* 72 and 42 for d<sub>4</sub>-furan. The relative response factor of furan with respect to d<sub>4</sub>-furan was  
171 calculated daily by analyzing a standard solution. For each run, analyses were made in duplicate. Furan concentration  
172 was expressed as ng/g of dry matter.

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174 Colour analysis

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176 Colour analysis was carried out on five different points of sample surface using a tristimulus colorimeter (Chromameter-  
177 2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-400 measuring head. The instrument was standardized  
178 against a white tile before measurements. Colour was expressed in L\*, a\* and b\* scale parameters and a\* and b\* were  
179 used to compute the hue angle ( $\tan^{-1} b^*/a^*$ ) (Clydesdale 1978).

181 Total solid content determination

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183 Total solid content was determined by gravimetric method by drying the samples in a vacuum oven (1.32 kPa) at 75 °C  
184 until a constant weight.

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186 Lipid extraction

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188 Lipids were extracted following the methodology described by Kristensen et al. (2000). About 10 g sample was  
189 transferred to a 100 mL centrifuge tube and 30 mL of isooctane was added. The sample was homogenized using a high  
190 speed homogenizer (D125, Ika-Werke, Staufen, Germany) at 9,000 rpm for 2 min. The blend was then transferred to an  
191 Erlenmeyer flask and added with 30 mL of isooctane, 30 mL of methanol and 60 mL of ethylic ether. Subsequently, the  
192 mixture was stirred at room temperature at 700 rpm for 20 min and then let statically settle. The liquid part was  
193 transferred to another Erlenmeyer flask and added with anhydrous sodium sulphate. The supernatant was filtered  
194 through Whatman n. 1 filter paper and evaporated using a rotary evaporator (Laborata 4001, Heidolph, Schwabach,  
195 Germany) at 120 rpm and 50 °C, decreasing the pressure from 900 mbar to 15 mbar in 30 min. Finally, the lipid fraction  
196 was transferred to a 10 mL vial, saturated with nitrogen, sealed and stored at -30 °C until analyses were performed.

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198 Peroxide value determination

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200 Peroxide value was determined following the method of Shanta and Decker (1994). Lipid samples (0.016 g) were  
201 weighted into 10 mL vials, added with 2.8 mL of methanol/butanol solution (2:1 v/v) and vortexed until the lipid  
202 fraction was dissolved. Subsequently, 0.015 mL of ferrous ion solution (prepared through the mixture of 0.033 M BaCl<sub>2</sub>  
203 and 0.036 M FeSO<sub>4</sub>) and 0.015 mL of 3.94 M ammonium thiocyanate were added in the vial and vortexed for 5 s. After  
204 20 min of incubation at room temperature, absorbance was measured at 510 nm with a UV-2501 PC spectrophotometer  
205 (Shimadzu, Kyoto, Japan) coupled with a CSP-Controller thermostat (Shimadzu, Kyoto, Japan) and a CPS-240A cell  
206 positioner (Shimadzu, Kyoto, Japan). Data were acquired using a UV-Probe v.2.31 (Shimadzu, Kyoto, Japan) software.  
207 Sample absorbance was corrected using a blank sample prepared mixing all the reagents except for the extracted lipids  
208 and incubated for 20 min at room temperature. The peroxide value (PV) expressed as milliequivalents of oxygen per  
209 kilogram of fat was calculated by using the following equation:

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$$PV = \frac{(A_s - A_b) \cdot m}{55.84 \times m_0 \times 2} \quad \text{eq. 2}$$

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211 where  $A_s$  is the absorbance of the sample,  $A_b$  is the absorbance of the blank,  $m$  is the slope of the calibration curve of  
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212  $\text{Fe}^{3+}$  concentration vs absorbance,  $m_0$  is the mass in grams of the sample and 55.84 is the atomic weight of iron.

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6 214 Rheology

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10 216 A Stresstech Rheometer (Reologia Instruments AB, Lund, Sweden) equipped with a 40 mm parallel-plate geometry and  
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12 217 application software Stresstech v. 4 was used. The temperature was kept at 20 °C by using a circulating coolant  
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14 218 connected to a thermostat. An aliquot of monoglyceride-flaxseed oil-water gel was placed on the measuring plate and  
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16 219 the measuring gap was set at 2 mm. To determine the linear viscosity range for the samples, dynamic stress sweep  
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18 220 measurement at a frequency of 1 Hz from 0.1 Pa to 100 Pa was conducted at 20 °C.  $G'$  and  $G''$  moduli were obtained for  
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20 221 a frequency scan from 0.1 to 10 Hz using a fixed stress value included in the linear viscoelastic region. Measurements  
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22 222 were carried out in duplicate on two replicated experiments.

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26 224 Firmness

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30 226 Biscuit firmness was measured using an Instron 4301 (Instron LTD, High Wycombe, UK). The instrumental settings  
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32 227 and operations were accomplished using the software Automated Materials Testing System (version 5, Series IX,  
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34 228 Instron LTD, High Wycombe, UK). The instrument was equipped with a 5 kN load cell. Biscuits were positioned on  
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36 229 two bars and broken by applying a cross-head speed of 5 mm/s to the blade edge. Firmness was defined as the  
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38 230 maximum force required to break the biscuit. From each baking experiment, six biscuits were analyzed.

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42 232 Polynomial equations and statistical analysis

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46 234 Modelling was aimed at describing the variation of water content, colour, peroxide value, acrylamide, and furan  
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48 235 concentrations as a function of the variables of the central composite design. In particular, a 3 factors face centred  
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50 236 central composite design was used, here after called CCF. The three considered factors were baking temperature, time  
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52 237 and pressure. For each factor, extreme, lower and upper values were identified and combined to form the factorial part  
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54 238 of the design (8 factorial points). To complete the CCF, 6 axial points (combinations of the extreme value of one factor  
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56 239 and the intermediate level for the others) and 1 central point (combination of the intermediate values of the three factors)  
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58 240 were defined.

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241 All the factorial and axial points were replicated once, while the central point was replicated 6 times. The full set of  
242 sampling points is reported in Table 1. As pressure values of different magnitude were considered, they were expressed  
243 in logarithmic scale for data modelling. A software package (Statistica for Windows v. 10, StatSoft, Inc.) was used to fit  
244 the second order response surface to the observed data according to the following equation:

$$y = B_0 + \sum_{i=1}^k B_i x_i + \sum_{i=1}^k B_{ii} x_i^2 + \sum_{j>i \geq 1}^k B_{ij} x_i x_j \quad \text{eq. 3}$$

247 where  $B_0$  is a constant, and  $B_i$ ,  $B_{ii}$ ,  $B_{ij}$  are regression coefficients of the model,  $x_i$  and  $x_j$  are the independent variables in  
248 coded values, and  $k$  is the number of factors.

249 Shapiro-Wilk test was used to evaluate normality of the data, while the possible presence of outliers and the  
250 homogeneity of variance were evaluated by residual analysis. Goodness of fit was measured with the adjusted  
251 determination coefficient ( $R^2_{\text{adj}}$ ).  $p$ -values for the coefficients of the response surface were defined using standard  $t$ -test.

252 Three-dimensional surface plots were drawn to illustrate the effects of the considered factors on the responses. To this  
253 purpose, the values of the response were plotted on the  $z$ -axis against the two most relevant factors, keeping the third  
254 one fixed to a constant value (the central one).

## 257 **Results and discussion**

### 259 Hydrogel formulation and its incorporation in the dough

260 To incorporate flaxseed oil in the dough, a monoglyceride-flaxseed oil-water gel, hereafter called hydrogel, was  
261 developed. A maximum amount of 47.6% (w/w) of flaxseed oil was incorporated into the gel. As expected, the flaxseed  
262 oil-based hydrogel presented gel-like properties, the  $G'$  modulus being higher than the  $G''$  modulus ( $8.88 \pm 0.11$  kPa and  
263  $2.46 \pm 0.14$  kPa, respectively), in agreement with the literature for monoglyceride-oil-water systems (Batte et al. 2007;  
264 Calligaris et al. 2010). As well documented in the literature, the hydrogel elasticity is attributable to the monoglycerides  
265 that, while self-assembling, may form a network of crystallized vesicles containing oil (Batte et al. 2007). The hydrogel  
266 was added to the dough formulation at 29% on total weight and its performance visually evaluated and compared with a  
267 control biscuit containing palm oil. After baking at 180 °C for 20 min at atmospheric pressure, a hydrogel containing  
268 biscuit regular in shape and colour ( $L^* = 65 \pm 2$  and hue angle =  $74 \pm 1$ , and  $L^* = 69 \pm 1$  and hue angle =  $78 \pm 1$ , for the  
269 hydrogel and palm oil containing biscuits, respectively) was obtained. The firmness of the hydrogel containing biscuit  
270 was slightly lower than that of the palm oil containing control sample ( $38 \pm 8$  N and  $52 \pm 10$  N, respectively), although

272 they did not differ significantly in water content (< 3% w/w). These results indicate that the use of the hydrogel allowed  
273 biscuits with desired quality to be obtained, in agreement with previous findings (Goldstein and Seetharaman 2011;  
274 Anese et al. 2011; Manzocco et al. 2012b). Therefore, the hydrogel was used to further prepare omega-3 PUFAs  
275 enriched samples to be subjected to baking according to the CCF.

#### 277 Identification of baking conditions at reduced pressure of omega-3 PUFAs enriched biscuits

278  
279 Table 2 shows the water content, hue angle, peroxide value as well as acrylamide and furan concentrations of biscuits  
280 containing the hydrogel subjected to baking under different conditions according to the CCF.

281 The regression coefficients and their relative analysis of variance of the polynomial models for the dependent variables  
282 are presented in Table 3.  $R^2_{adj}$  values for the responses were higher than 0.882.

283 As it can be observed in Table 2, all factor combinations, except that including the highest factors values (run 4),  
284 allowed furan accumulation to be prevented. In fact, in contrast with the biscuit baked at atmospheric pressure and 197  
285 °C for 45 min that presented a furan concentration of 174 ng/g, furan levels were always lower than 32 ng/g and in most  
286 cases below the quantification limit (10 ng/g) for this molecule. As a consequence, it was not possible to find an  
287 appropriate model to describe these data.

288 The results showed that temperature as well as the linear and quadratic terms of log pressure had a significant effect on  
289 moisture content, showing *p*-values lower than 0.001, 0.01, and 0.001, respectively. To evaluate the effects of the  
290 independent variables on the dependent ones and to predict the optimum values of each variable for  
291 maximum/minimum yield, three-dimensional response surface plots were generated. Fig. 1 shows the response surface  
292 plot relevant to the effect of temperature and pressure on water content changes occurring in the biscuits during baking.  
293 As expected, the lowest moisture contents were achieved at the highest temperature and pressure conditions. Results  
294 also show that baking at reduced pressure slightly favoured moisture removal. This can be attributable to a stripping  
295 effect towards water, that was promoted by the low pressure inside the oven. Moreover, Fig. 1 shows that, in our  
296 experimental conditions, the minimum water content was obtained at pressure values of 0.15 kPa (corresponding to 3.6  
297 Log Pa). It is noteworthy that a 3% moisture, that is the highest water content acceptable for biscuits, was also achieved  
298 by 40 min baking at the lowest temperature (150 °C) and pressure (0.15 kPa).

299 As known colour is an important quality parameter for biscuits. Colour development is the result of the formation of  
300 brown polymers (i.e. melanoidins) in the advances stages of non-enzymatic browning reactions. As colour development  
301 has been associated to acrylamide formation, light brown is considered an acceptable colour for biscuits (Food Drink  
302 Europe 2013). Biscuits colour changes were assessed by means of colour measurements and expressed as hue angle. A

303 decrease in hue angle is an index of browning development. According to the results shown in Table 3, temperature and  
304 pressure as both linear and quadratic terms, significantly affected the hue angle of the biscuits, although the influence of  
305 pressure quadratic term was lower than that of the other factors. Fig. 2 shows the response surface plot of the effect of  
306 temperature and pressure on the hue angle of the biscuits subjected to baking. Baking at the lowest temperature and  
307 pressure conditions led to the least coloured biscuits, while lower hue angle values (i.e. the higher colour development)  
308 were achieved at temperatures higher than 190 °C at both high and low pressure values. It is noteworthy that baking at  
309 ambient pressure allowed colour development to occur at lower temperature as compared to the vacuum process. These  
310 results suggest that moisture and colour values acceptable for biscuit production can be actually achieved at reduced  
311 pressure conditions.

312 As already pointed out, due to its chemical feature, omega-3 PUFAs are very susceptible to oxidative reactions, that  
313 may be favoured by the high baking temperature. To study the influence of the independent variables on the oxidation  
314 of omega-3 fatty acids, the peroxide value of the biscuit lipid fraction was measured (Table 2). As shown in Table 3,  
315 temperature and pressure as linear and interactive terms were the major factors influencing this parameter ( $p < 0.001$ ),  
316 while time and the interaction between time and temperature had a lower effect ( $p < 0.05$ ). As shown in Fig. 3, the  
317 maximum peroxide value was achieved at the highest temperature and pressure values. No peroxide formation was  
318 detected in biscuits subjected to baking at any temperature when the pressure was kept at intermediate or low values. It  
319 can be suggested that when baking was performed at reduced pressure conditions, the low oxygen concentration present  
320 inside the oven would prevent oxidative reactions to occur.

321 The effect of the process variables on acrylamide formation in the biscuits was also evaluated. As already stated,  
322 considerably high amounts of this heat-induced toxic molecule can form upon baking of cereal products (Claus et al.  
323 2008). In particular, acrylamide formation proceeds faster in the final steps of baking in correspondence of low moisture  
324 contents (Bråthen and Knutsen 2005) along with brown development (Gökmen and Şenyuva 2006). The ANOVA  
325 showed that all the process variables, except the quadratic term of time, affected the formation of the toxic molecule,  
326 although with different significance (Table 3). Temperature and pressure as linear and interactive terms were the  
327 process variables most affecting the acrylamide yields, as indicated by their smaller  $p$ -values. The effects of the  
328 interactions between pressure and temperature on acrylamide formation are shown in Fig. 4. A maximum acrylamide  
329 level was attained at the highest pressure and temperature values. Acrylamide levels lower than 500 ng/g, that is the  
330 maximum recommended concentration in biscuits (EFSA 2013), were formed at any temperature when pressure was  
331 reduced from atmospheric to intermediate or low values.

332 Overall, these results suggest that, within the margins of the present study, baking at high temperature and reduced  
333 pressure allowed to obtain biscuits with acceptable water content and colour, while minimizing omega-3 PUFAs

334 degradation by oxidative reaction as well as acrylamide and furan formation. In particular, the combination 174 °C-3.99  
335 kPa-45 min allowed biscuits responding to these characteristics to be obtained.

336 In order to compare the impact of pressure on non-enzymatic browning reaction independently of the combination of  
337 temperature and time experienced by the sample during baking, data relevant to hue angle and acrylamide (Table 2)  
338 were further elaborated. In particular, they were plotted against the thermal effect  $F$ , that is the time-temperature  
339 combination received by the dough at each baking time (Fig. 5). Table 4 shows the rate constants computed from the  
340 slopes of the linear regression of hue angle and acrylamide concentration of short dough biscuits subjected to baking  
341 under different pressure conditions vs  $F$ . The computed rate constants showed statistical differences from 0 ( $p<0.05$ ).  
342 The pseudo zero order rate constant of hue angle in the biscuits baked at atmospheric pressure was higher (i.e.  
343 indicating a slower browning development) than those found for the biscuits obtained at lower pressures. In particular,  
344 discrepancies in hue angle among the biscuits were higher at low  $F$  values and decreased with the increasing of  $F$ ,  
345 becoming similar at approximately 38 min (i.e. the highest  $F$  value received by the samples). Moreover, no significant  
346 differences between the rate constant computed for the baking processes carried out at 3.99 and 0.15 kPa were found  
347 ( $p>0.05$ ). This lower value of hue angle rate constant (i.e. higher rate of colour development) at low pressure can be  
348 attributed to faster water removal due to the low pressure inside the oven (Table 2). In addition, as exemplified in Table  
349 4, the rate of acrylamide formation for increasing  $F$  values was greater for the biscuits baked at ambient pressure than  
350 for samples cooked at reduced pressure. Similar results have been previously found for coffee subjected to roasting  
351 under low pressure conditions (Anese et al. 2014). These data seem to suggest that (a) lower acrylamide is formed at  
352 reduced pressure conditions or (b) as soon as the toxic molecule is generated, the low pressure generated inside the oven  
353 would promote acrylamide removal, thus preventing its accumulation. Moreover, in the latter case, as acrylamide is a  
354 water soluble molecule, it is likely that its removal occurs along with that of water. Similar mechanisms can be  
355 suggested for furan. In this case, all the baking conditions at reduced pressure were effective in keeping furan levels  
356 below the quantification limit (Table 2). According to the removal mechanism, it can be suggested that as soon as furan  
357 is formed, it is almost quantitatively removed due to its higher volatility as compared with acrylamide.

## 359 **Conclusions**

360 The results of the present study indicate that baking at high temperature and reduced pressure allowed to obtain biscuits  
361 with acceptable water content and colour, while minimizing omega-3 PUFAs degradation by oxidative reaction as well  
362 as acrylamide and furan levels. In particular, results of the experimental plan showed that the combination 174 °C-3.99  
363 kPa-45 min resulted effective in producing biscuits with acrylamide concentration and peroxide value below 100 ng/g

365 and 2 meqO<sub>2</sub>/kg<sub>fat</sub> respectively, as well as furan at negligible levels. As compared with the conventionally baked sample  
366 (200°C-101.3 kPa-35 min), these baking conditions led to an acceptable reduction in brown development. It is  
367 noteworthy that this is in line with the indications given by the Food Drink Europe association stating that producing  
368 lighter coloured biscuits, without increasing the moisture content, could represent a strategy to reduce acrylamide  
369 content. Moreover, as the application of baking at low pressure conditions may be responsible for the removal of  
370 desired flavor compounds, sensory analysis has to be performed.

371 Although further research should be conducted at pilot and industrial scale to find optimum process conditions, these  
372 results suggest that baking under reduced pressure could have a great economic impact, due to the large diffusion of this  
373 food category; not only the conventional bakery products but also the functional ones are becoming very popular.

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479 **Figure captions**

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481 **Fig. 1** Response surface plot showing the effect of baking temperature and pressure on water content of short dough  
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482 biscuits. A constant value (central point) was imposed to the third independent variable (time) of the CCF

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1484 **Fig. 2** Response surface plot showing the effect of baking temperature and pressure on hue angle of short dough  
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1485 biscuits. A constant value (central point) was imposed to the third independent variable (time) of the CCF

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1487 **Fig. 3** Response surface plot showing the effect of baking temperature and pressure on peroxide value of the lipid  
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1488 fraction of short dough biscuits. A constant value (central point) was imposed to the third independent variable (time) of

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2491 **Fig. 4** Response surface plot showing the effect of baking temperature and pressure on acrylamide concentration of  
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2492 short dough biscuits. A constant value (central point) was imposed to the third independent variable (time) of the CCF

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3494 **Fig. 5** Hue angle and acrylamide concentration of short dough biscuits subjected to baking under reduced pressure  
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3495 conditions as a function of the thermal effect  $F$

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497 **Table 1** Combinations of time, temperature and pressure of different runs of a three factors face centred central  
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 498 composite design  
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3	4	5	6	7
Run	Temperature	Pressure	Time	
	(°C)	(Log Pa)	(min)	
8				
9				
10	1	150	5.00	45
11				
12	2	150	5.00	35
13				
14	3	197	5.00	35
15				
16	4	197	5.00	45
17				
18	5	150	2.17	45
19				
20	6	150	2.17	35
21				
22	7	197	2.17	45
23				
24	8	197	2.17	35
25				
26	9	174	2.17	40
27				
28	10	174	5.00	40
29				
30	11	174	3.60	45
31				
32	12	174	3.60	35
33				
34	13	197	3.60	40
35				
36	14	150	3.60	40
37				
38	15	174	3.60	40
39				
40	16	174	3.60	40
41				
42	17	174	3.60	40
43				
44	18	174	3.60	40
45				
46	19	174	3.60	40
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48	20	174	3.60	40
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501 **Table 2** Experimental results  $\pm$  standard deviation of a 3 factors face centred central composite design

Run	Water content (% w/w)	Hue angle ( $\tan^{-1}b^*/a^*$ )	Peroxide value (mEq O <sub>2</sub> /kg <sub>fat</sub> )	Acrylamide (ng/g)	Furan (ng/g)
1	6.03 $\pm$ 0.16	79.2 $\pm$ 0.3	3.07 $\pm$ 0.03	125.7 $\pm$ 4.4	10 $\pm$ 2
2	9.06 $\pm$ 0.13	81.3 $\pm$ 0.5	2.40 $\pm$ 0.01	51.3 $\pm$ 1.9	11 $\pm$ 2
3	2.49 $\pm$ 0.01	71.8 $\pm$ 0.5	20.12 $\pm$ 0.71	419.2 $\pm$ 18.8	27 $\pm$ 4
4	0.48 $\pm$ 0.06	67.7 $\pm$ 2.3	10.92 $\pm$ 3.60	709.3 $\pm$ 31.5	174 $\pm$ 24
5	5.14 $\pm$ 0.01	90.9 $\pm$ 0.4	3.07 $\pm$ 0.02	10.0 $\pm$ 1.1	20 $\pm$ 3
6	5.09 $\pm$ 0.02	90.8 $\pm$ 0.5	2.40 $\pm$ 0.01	9.0 $\pm$ 0.7	9 $\pm$ 1
7	0.40 $\pm$ 0.02	67.8 $\pm$ 2.7	2.30 $\pm$ 0.08	225.7 $\pm$ 6.4	9 $\pm$ 2
8	0.54 $\pm$ 0.07	73.8 $\pm$ 2.6	2.62 $\pm$ 0.02	199.6 $\pm$ 4.5	12 $\pm$ 2
9	1.59 $\pm$ 0.12	90.8 $\pm$ 0.6	2.60 $\pm$ 0.01	19.2 $\pm$ 1.6	9 $\pm$ 2
10	5.25 $\pm$ 0.09	75.1 $\pm$ 0.6	5.15 $\pm$ 0.21	220.5 $\pm$ 6.9	9 $\pm$ 2
11	0.82 $\pm$ 0.25	85.7 $\pm$ 2.3	1.75 $\pm$ 0.02	75.1 $\pm$ 2.4	9 $\pm$ 1
12	1.31 $\pm$ 0.10	90.1 $\pm$ 2.0	4.72 $\pm$ 0.59	42.7 $\pm$ 0.6	9 $\pm$ 1
13	0.38 $\pm$ 0.04	69.3 $\pm$ 3.2	6.00 $\pm$ 0.41	255.5 $\pm$ 3.5	11 $\pm$ 2
14	2.42 $\pm$ 0.08	92.0 $\pm$ 0.2	2.57 $\pm$ 0.01	7.8 $\pm$ 1.2	9 $\pm$ 2
15	0.87 $\pm$ 0.16	88.1 $\pm$ 1.9	4.05 $\pm$ 0.05	51.5 $\pm$ 2.5	10 $\pm$ 1
16	1.47 $\pm$ 0.02	91.6 $\pm$ 1.2	1.92 $\pm$ 0.02	86.0 $\pm$ 3.0	9 $\pm$ 1
17	0.71 $\pm$ 0.05	90.0 $\pm$ 1.5	1.62 $\pm$ 0.02	31.9 $\pm$ 1.0	10 $\pm$ 2
18	0.53 $\pm$ 0.01	88.1 $\pm$ 4.4	2.02 $\pm$ 0.07	77.6 $\pm$ 0.8	9 $\pm$ 2
19	0.83 $\pm$ 0.03	87.9 $\pm$ 3.6	2.72 $\pm$ 0.05	94.6 $\pm$ 3.5	31 $\pm$ 5
20	1.23 $\pm$ 0.19	90.4 $\pm$ 1.6	2.55 $\pm$ 0.00	42.8 $\pm$ 2.0	13 $\pm$ 3

506 **Table 3** Regression coefficients of the models for water content, hue angle, peroxide value and acrylamide

Variable	Water content	Hue angle	Peroxide value	Acrylamide
Intercept	40.204	-219.914	70.835	7538.511
Temp	-0.305***	4.108***	-0.766***	-61.552***
Temp <sup>2</sup>	0.0006	-0.012**	0.003	0.146**
LogP	-2.371**	0.551**	-13.007***	-766.073***
LogP <sup>2</sup>	1.174***	-2.199*	0.605	38.846*
Time	0.053	-0.301	0.592*	-83.160**
Time <sup>2</sup>	-0.0001	0.022	0.022	0.324
Temp x LogP	0.010	0.062	0.097***	2.039***
Temp x Time	0.0009	-0.011	-0.012*	0.256*
LogP x Time	-0.087	0.038	-0.156	5.930**
R <sup>2</sup> adj	0.882	0.884	0.871	0.953

507 \* :  $p < 0.05$ ; \*\* :  $p < 0.01$ ; \*\*\* :  $p < 0.001$

508 **Table 4** Rate constants  $\pm$  95% confidence interval errors computed from the slopes of the linear regression of hue angle  
 509 and acrylamide concentration of short dough biscuits subjected to baking under different pressure conditions vs the  
 510 thermal effect

Parameter	Pressure (kPa)	Rate constant ( $\tan^{-1}b^*/a^*$ $\text{min}^{-1}$ ; ng/g $\text{min}^{-1}$ )	$R^2_{\text{adj}}$
Hue angle	101.3	$-0.36 \pm 0.15^a$	0.936
	3.99	$-0.77 \pm 0.17^b$	0.927
	0.15	$-0.72 \pm 0.27^b$	0.947
Acrylamide	101.3	$17.29 \pm 6.17^{a'}$	0.952
	3.99	$7.91 \pm 1.97^{b'}$	0.904
	0.15	$7.05 \pm 2.66^{b'}$	0.960

511 <sup>a,b</sup>: For each parameter considered, significant difference is indicated by different letters ( $p < 0.05$ )

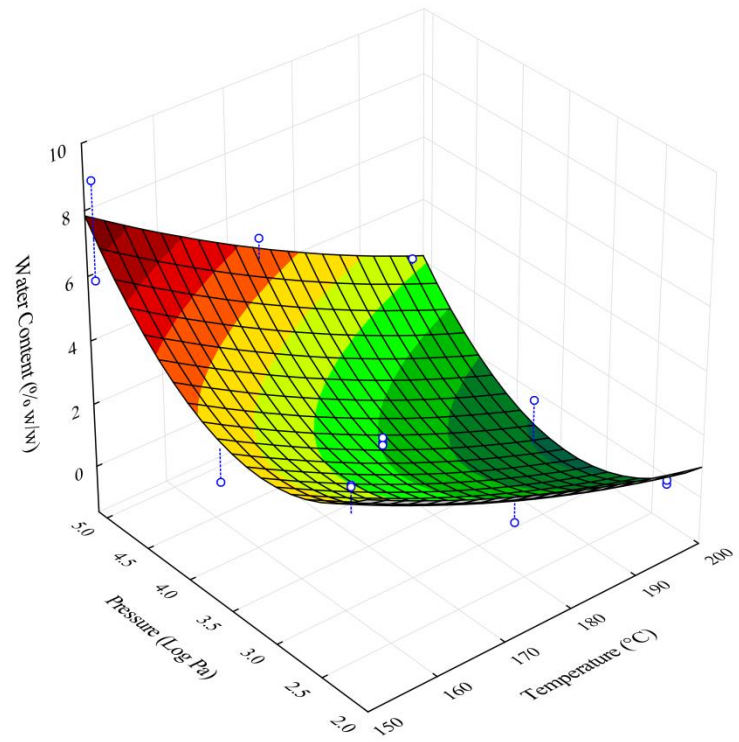


Fig 1

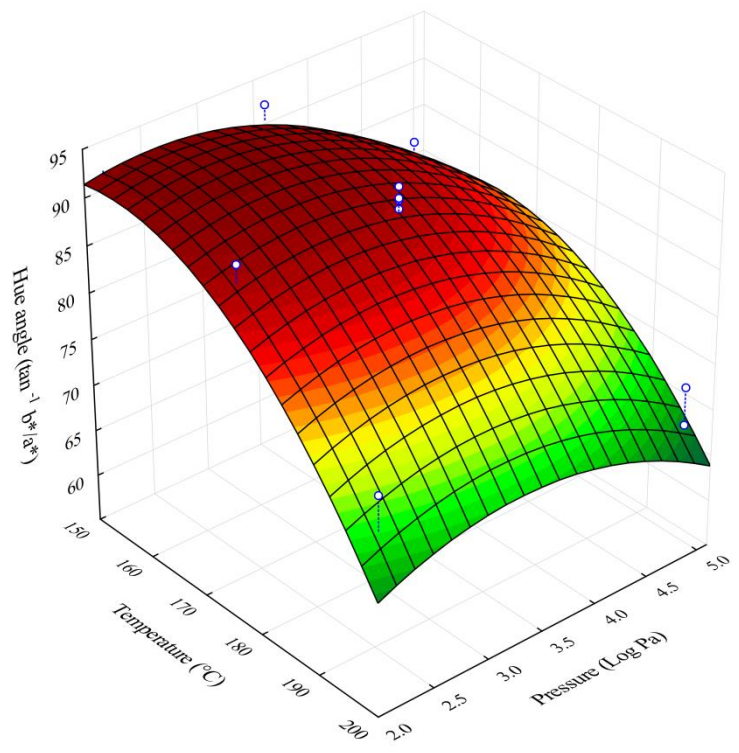


Fig 2



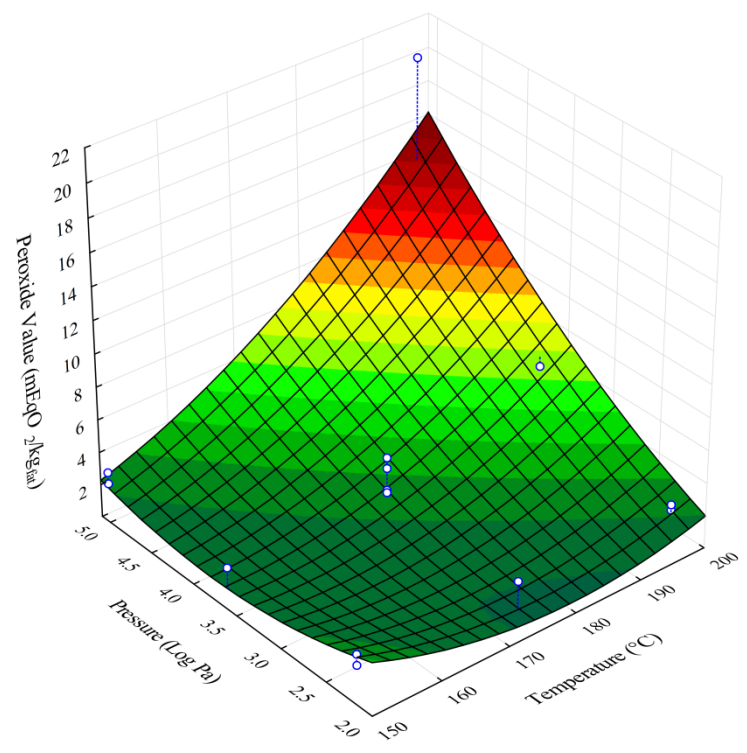


Fig 3

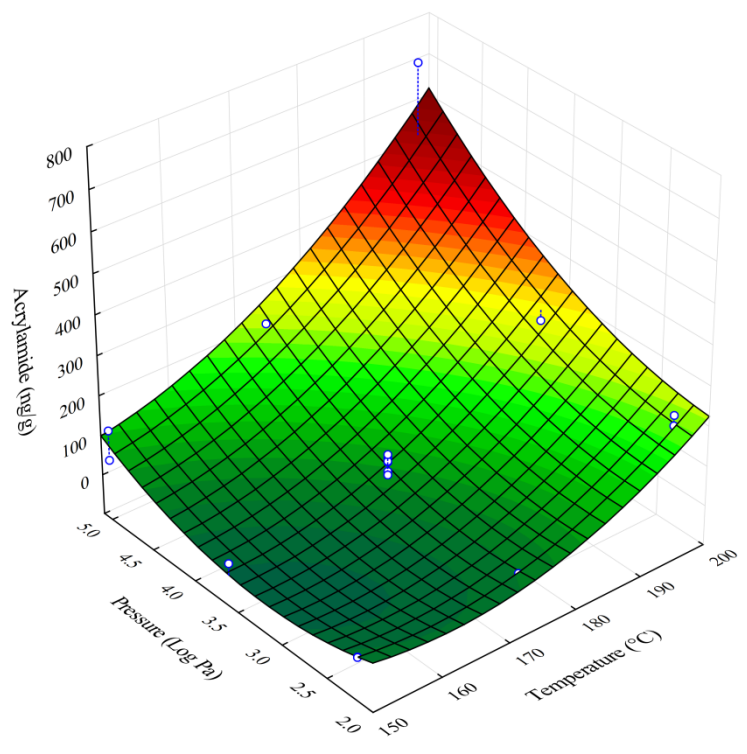


Fig 4

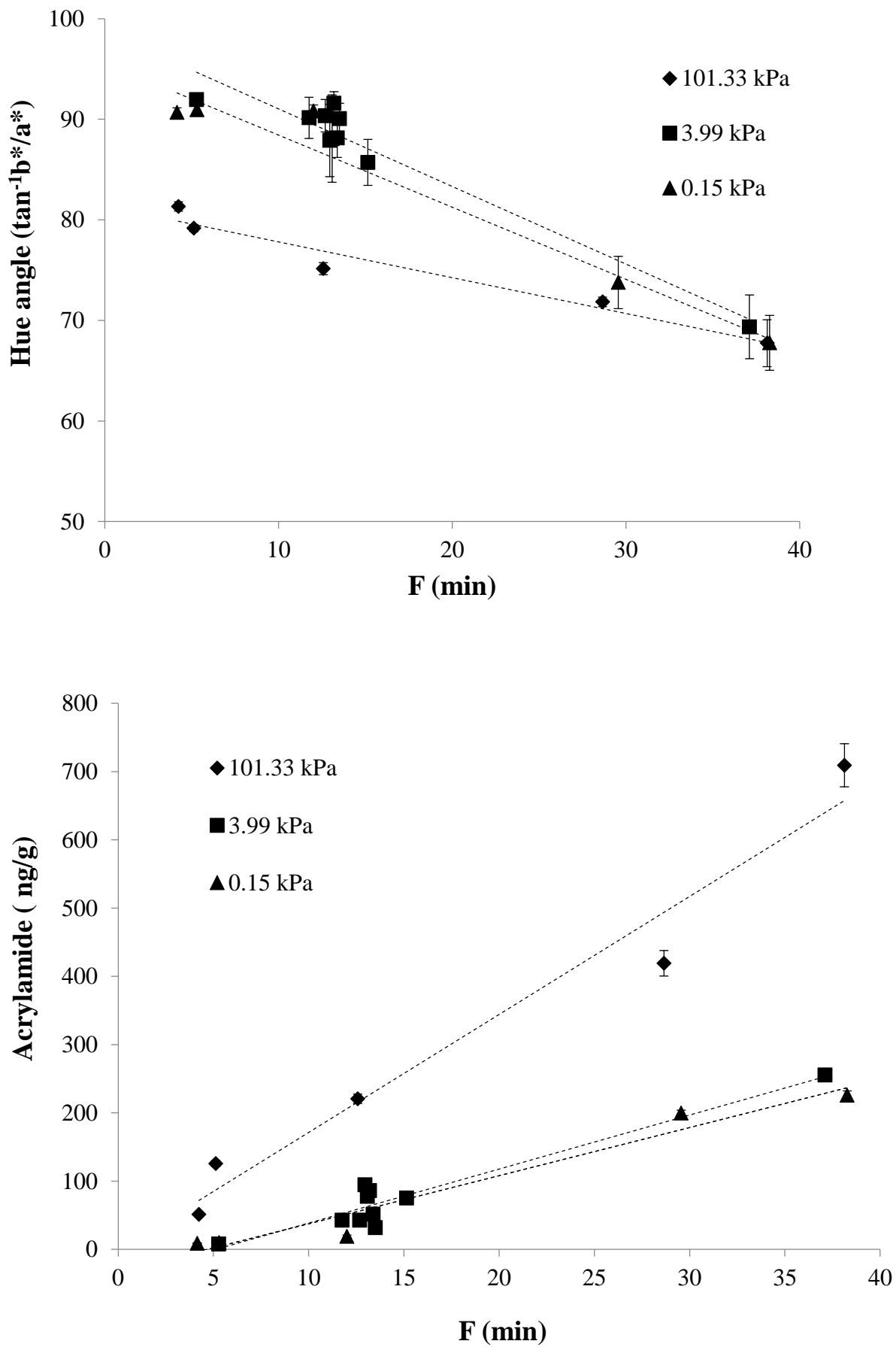


Fig. 5