1 **In vitro starch digestibility and fate of crocins in pasta enriched with saffron extract**

2 Armellini, R.[1,2], Peinado, I.*[3], Asensio-Grau, A.[3], Pittia, P.[2], Scampicchio, M. [1], Heredia, A. [3]

3 & Andres, A.[3]

4

5 Rita Armellini, [1]Free University of Bolzano, Faculty of Science and Technology, 39100,

6 Bolzano, IT & [2]University of Teramo, Faculty of Bioscience and Technology for Food

7 Agriculture and Environment, 64100 Teramo, IT. rita.armellini@gmail.com

8 *Irene Peinado, [3]Research Institute for Food Research and Development, Universitat

9 Politècnica de València, P.O. Box 46022, Valencia, Spain.   Corresponding author:

10 irpeipar@gmail.com; tel: +34 963877365.

11 Andrea Asensio Grau, [3]Research Institute for Food Research and Development, Universitat

12 Politècnica de València, P.O. Box 46022, Valencia, Spain. anasgr@upv.es

13 Paola Pittia, [2]University of Teramo, Faculty of Bioscience and Technology for Food

14 Agriculture and Environment, 64100 Teramo, IT. ppittia@unite.it

15 Matteo Scampicchio, [1]Free University of Bolzano, Faculty of Science and Technology, 39100,

16 Bolzano, IT. matteo.scampicchio@unibz.it

17 Ana Heredia, [3]Research Institute for Food Research and Development, Universitat Politècnica

18 de València, P.O. Box 46022, Valencia, Spain. anhegu@tal.upv.es

19 Ana Andrés, [3]Research Institute for Food Research and Development, Universitat Politècnica

20 de València, P.O. Box 46022, Valencia, Spain. aandres@tal.upv.es

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Abstract

This work aims to study the effect of the addition of saffron extract on fresh pasta in-vitro digestibility. Fresh pasta was formulated with different concentrations of saffron extracts (0.2 and 0.4 %w/w), cooked at two different times (1.5 and 3 min), and in vitro digested (oral, gastric and intestinal stages). Oil was added to pasta before digestion to evaluate the presence of lipids on starch and crocin bioaccessibility. Saffron enrichment and oil addition slowed down the digestion of starch, thus, decreasing the glycemic index of pasta. Concentration of saffron and oil addition contributed to crocin release in the digestion fluids, with the opposite effect of cooking time. Isomerization from *trans* to *cis* was enhanced by both, cooking and oil addition. Bioaccessibility of total crocins varied from 2.9±1.1, to 97±3 %. Finally, the *trans:cis* isomers distribution was only close to 50:50 in enriched-pasta cooked during 3 min or with oil addition.

Chemical Compounds

Crocin I (Pubchem CID: 5281233); *trans*-crocetin (b-D-glucosyl)-(b-D-gentiobiosyl) ester (Pubchem CID: 9940690); Crocetin Digentiobiosyl Ester (Pubchem CID: 44630212); Safranal (Pubchem CID: 61041).

Key-words: saffron, pasta, oil, in vitro digestion, starch, crocin bioaccessibility

1. Introduction

Pasta is a worldwide known food product. It is an important carbohydrate source, composed mainly of starch and low content of fats, proteins, dietary fibre, minerals, vitamins and phenolic compounds. Starch, the most important digestible polysaccharide in human nutrition usually accounts for 20-50 % of the total energy intake. It is made up of two different glucose polymers, amylose (15-20 %) and amylopectin (80-85 %), and it is mainly derived from plant seeds such as wheat, maize, rice, oats and rye. Starch is a major source of glucose that appears at relatively high concentrations in blood circulation during digestion, playing a predominant role in the human diet as it contributes to the glycaemic index (Bohn et al., 2017). The glycaemic index (GI) is associated with the presence and concentration of carbohydrates in a particular food, and it indicates their effect on a person's blood glucose level. Acute increments in postprandial plasma glucose and insulin levels after eating foods rich in carbohydrate (high glycaemic response), may increase the risk of metabolic diseases such as type 2 diabetes, cardiovascular disease, and obesity (Kim et al., 2008). Therefore, low glycemic responses are considered favorable to health, and ways are being sought to reduce the glycemic impact of food carbohydrates (Kim et al., 2008). Moreover, it is known that a series of factors including the type of carbohydrate, physical entrapment of the carbohydrate within the food, as well as fat and protein content of the food and organic acids or salt content of the meal may affect glycemic index (Bohn et al., 2017). Pasta is a popular and simple carbohydrate-based food with a relative low glycemic response (Singh, Dartois, & Kaur, 2010) that derives from its composition and structural properties determined by the wheat flour and processing.

In order to enhance nutritional value of pasta, several studies have focused on the possibility of adding functional ingredients into it (Bustos, Perez, & León, 2013; Fiorda, Soares, da Silva, Grosmann, & Souto, 2013). Design and development of fortified formulated bakery products,

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68   is one of the latest trends in the specific industry sector with a growing interest due to their

69   improved nutritional properties.

70   Among the wide variety of functional ingredients with potential health benefits, saffron, a spice

71   derived from the dried stigmas of *Crocus sativus* L. flowers, has received increased attention

72   in the past two decades in both, cookery and food industry, due to its colouring properties,

73   pleasant bitter taste and alluring aroma (Serrano-Díaz, Sánchez, Maggi, Carmona, & Alonso,

74   2011). It owes its sensory properties mostly to the presence of three carotenoid derivates,

75   mainly synthesized during flowering, but also during processing, which are crocins,

76   picrocrocin and saffranal, responsible of the saffron colour, flavour and aroma, respectively.

77   These secondary metabolites are produced by oxidative cleavage of zeaxanthin, followed by

78   oxidative modifications and glycosylation (Namin et al., 2009). Crocetin digentiobiose ester

79   ($C_{44}H_{64}O_{24}$), a natural and food-grade colouring agent, is considered one of the major bioactive

80   constituents of saffron and has a wide spectrum of biological activities including antigenotoxic

81   and cytotoxic effects, antioxidant, anti-inflammatory, anti-atherosclerosis, anti-diabetic,

82   hypotensive, hypolipidaemic, hypoglycemic and antidepressant (Rahaiee, Moini, Hashemi, &

83   Shojaosadati, 2015). However, in general, crocins have low stability and lose most of their

84   functionality during processing and storage after exposure to heat, oxygen, light, acidic

85   environment and/or due to presence of additives (Maggi et al., 2009).

86   In order to exert the target functionality in vivo, bioactive compounds need to be active after

87   digestion. The fraction of a bioactive compound released from the food matrix following

88   digestion, that is solubilised into the gut for intestinal uptake, is usually known as the

89   bioaccessible fraction (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014;

90   Rodríguez-Roque et al., 2015). The bioaccessibility of a particular compound depends, often

91   to a significant extent, on interactions with other dietary constituents (O'Brien, 2013). Besides

92   crocins and saffranal, saffron is also rich in many other bioactive compounds including

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93 phenols, that along with other phytochemicals, can be linked to macro-molecules via bonds of

94 various nature depending on their molecular and chemical characteristics. In fact, phenols have

95 been able to influence the starch digestibility both, in vitro and in vivo (Forester, Gu, &

96 Lambert, 2012). Thus, food matrix interactions between bioactives and macronutrients in

97 complex foods like pasta should be considered as potentially affecting the digestibility of the

98 macronutrients (i.e. starch, gluten) and, in turn, the bioaccessibility and bioavailability of the

99 micronutrients and bioactives.

100 The enrichment of pasta with other functional ingredients may have effects on its digestibility,

101 and, in particular, on that related to the starch fraction. That is why, in this study, the main goal

102 of this research was to study the effect of saffron extract addition (0.2 and 0.4 %w/w) on the

103 digestibility of starch as well as the fate and release of bioactive compounds and in particular,

104 crocins, of fresh pasta subjected to different cooking times (1.5 and 3 minutes). The impact of

105 the addition of olive oil to cooked pasta, and prior to digestion, on crocins bioaccessibility was

106 also evaluated.

107

108 **2. Materials and methods**

109 *2.1. Chemicals*

110 Crocin, saffranal, and methanol, were purchased from Sigma Sigma-Aldrich (Deisenhofen,

111 Germany). Ammonium bicarbonate, potassium dihydrogen phosphate, porcine pepsin (3200-

112 4500 U / mg), pancreatin (8 × USP) from porcine pancreas and bovine bile extract, were from

113 Sigma-Aldrich (Deisenhofen, Germany). Sodium carbonate hydrogen was purchased from

114 Scharlau (Barcelona, Spain). DNS (3-5' dinitrosalicylic acid) reagent, glucose, Na-K

115 tartrate/NaOH, ethanol, invertase and acetonitrile were purchased from Sigma-Aldrich

116 (Deisenhofen, Germany). Amyloglucosidase was from Megazyme (E- AMGDF, Megazyme,

117 Ireland). All solvents used for the determination of crocins were HPLC grade and the others of

118   analytical grade. Bidistilled water was used for chromatographic analysis (Milli-Q, Millipore

119   Corp., Bedford, MA). Standard solutions were stored at -20 ° C.

120   Saffron powders (Hacendado, Valencia) and wheat flour (Hacendado, Valencia) were

121   purchased in a local market. For pasta preparation and cooking, tap water of Valencia's

122   municipality (Valencia, Spain) was used.

123

124   ***2.2 Sample preparation***

125   *2.2.1. Pasta samples preparation*

126   All samples (with and without saffron) were prepared by mixing wheat flour and water (70:30

127   (w/w)). Different batches of pasta were made and each of them was made of ca. 250 g.

128   The following pasta formulations were prepared based on a previous study (Armellini et al.,

129   2018) as follows: control dough (C) made of wheat flour (165 g) and tap water (70 ml), (no

130   saffron); "saffron enriched pasta" was made of wheat flour (165 g) + 70 ml of a dispersion of

131   saffron powder in tap water (either 0.2 or 0.4 %w/w saffron in dough). All the ingredients were

132   mixed using a home professional equipment (Thermomix TM- 31, Vorwerk, Wuppertal,

133   Germany) for 2 min under 'dough mode'. The mix was left to settle for 5 minutes at room

134   temperature before to extrude it to a "pappardelle" shape (large and flat, width: 4 cm length:

135   24 cm) using a home scale pasta-shaper (Marcato SPA, Padova, Italy).

136   *2.2.2. Cooking of the pasta*

137   Aliquots of of pasta (60 g each) were cut into smaller pieces (8 cm x 4 cm) and cooked in

138   boiling tap water (1.5 L) (Armellini et al., 2018). Samples were removed from the boiling water

139   1.5 minutes after boiling started, that corresponded, based on preliminary tests, to the optimum

140   cooking time from the organoleptic point of view, as well as crocins preservation (Armellini et

141   al., 2018). Additionally, pasta enriched with 0.4% of saffron was also cooked for 3.0 min in

142   order to evaluate the effect of overcooking.

143 Samples were coded based on their saffron enrichment (0 (control), 0.2 or 0.4 % (w/w) and

144 cooking time (1.5 or 3.0 min) as follows: 0%-1.5' (control), 0.2%-1.5',0.4%-1.5', 0.4%-3').

145 After cooking, samples were drained and cooled by soaking them in cold water for 10 s; excess

146 water was removed by lightly patting the samples between paper towels. An aliquot of both

147 raw and cooked pasta corresponding to half of the sample was immediately used for moisture

148 measurements and in vitro digestion. The remaining part was freeze-dried (-45 ℃, 0.8 Pa 48

149 h, Telstar, Spain) and used for the remaining chemical analyses.

150 Data reported are referred to at least three different batches of pasta samples prepared in

151 different days. All measurements were performed in triplicate for each batch (n=9).

152

153 *2.3 In vitro digestion process*

154 The digestive process (oral, gastric and intestinal stages) and the simulated fluids (salival,

155 gastric and intestinal) were prepared according to the protocol proposed by Minekus et al.,

156 (2014), with slight modifications. The simulated salival fluid (SSF), the simulated gastric fluid

157 (SGF) and simulated intestinal fluid (SIF) were prepared daily from stock solutions. The

158 electrolyte composition of the different solutions is shown in **Table 1**. The enzymatic activity

159 was tested before each experiment following the protocol proposed by Carrière et al., (2000).

160 Each experiment was performed in triplicate. The *in vitro* digestion process was performed as

161 follows:

162 For the *oral stage,* pasta samples were mixed with SSF (1:1 w/v). Firstly, three healthy

163 volunteers, providing a signed consent form, chewed the tested pasta samples (5 g) 15 times

164 for approximately 15 seconds and then expectorated it into a 50 mL Falcon tube. After that,

165 the bolus was weighted again and the amount of SSF (pre-warmed at 37 ℃; pH 8) required to

166 complete the ratio (1:1 w/v) was added. The *gastric stage*, continued with the immediate

167 addition to the oral bolus of 10 mL of SGF, 5 μL of $CaCl_2$ (0.3 M) and a sufficient volume of

168   HCl (1M) to adjust the pH to 2.8 ± 0.1. Porcine pepsin had been added into the SGF to a

169   concentration of 25000 U pepsin / mL of SGF. Subsequently, the mixture was placed in a

170   thermostatically controlled chamber (Selecta, Spain) at 37 °C coupled to a shaker (Intelli-Mixer

171   RM 2, Elmi Ldt., Baltics and Russia) at a speed of 55 rpm and head-over-heels rotatory

172   movements. The total duration of this stage was 120 min. Every 30 min, the pH was measured

173   and re-adjusted (if necessary). For the *intestinal stage*, 20 mL of SIF were added to the gastric

174   chime (ratio 1: 1 (v/v)), pH was adjusted to 7.0 ± 0.1 with NaOH 1M, right after 120 min of

175   gastric stage. The final bile salts and pancreatin concentrations were 10 mM and 1260 LU

176   (Lipase Units or FCC), respectively, in each tube.

177   Furthermore, in order to evaluate the effect of the addition of a lipid component as excipient

178   on starch digestibility and bioacessibility of bioactive compounds, two samples (4 g) namely

179   the pasta formulated with 0.2 and 0.4 % w/w of saffron cooked for 1.5 min were individually

180   mixed with 1 g of olive oil, prior to the *oral stage*. These sample were coded as '0.2%-1.5'+oil'

181   and '0.4%-1.5'+oil'.

182   To stop the enzymatic reactions after the different sampling times along the gastric and

183   intestinal simulated digestion, the tubes were placed in ice for 10 min and afterwards

184   centrifuged at 1200 x G for 15 min, at 10° C, to separate the solid pellet and the liquid

185   supernatant, called 'micellar' phase. The pellet of each sample was used to determine the

186   matrix degradation index (MDI) while the micellar phase was divided into aliquots (1 mL) and

187   frozen at -40°C until analysis including starch determination and carotenoids. For the latter

188   analysis, samples were stored at -40 °C for 24 h before freeze-drying (-45 ºC, 0.8 Pa 48 h,

189   Telstar, Spain).

190 ***2.4. Analytical determinations***

191 *2.4.1 Matrix Degradation Index*

192 Matrix Degradation Index of each sample was determined from the proportion of solids that

193 were finely dispersed in the digestion juices. After centrifugation, the pellet collected from the

194 digestion tube was filtered on a metallic sieve (1.6 mm x 1.6 mm mesh) to separate out large

195 food particles. The drained liquid or micellar phase was collected and used for starch

196 determination as well as crocins analysis. The solid pasta particles were rinsed twice with 2

197 mL of digestion juice to remove any digested material and transferred to an aluminium dish

198 previously exactly weighted, placed in a forced air oven at 60 ºC for 48 h, and weighed again

199 to determine the mass of large pasta solids. The matrix degradation index (MDI) corresponds

200 to the proportion of pasta solids passing the metallic sieve (Asensio-Grau, Peinado, Heredia,

201 & Andrés, 2018; Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014; Lamothe, Rémillard,

202 Tremblay, & Britten, 2017).

203 *2.4.2. Digestible Starch*

204 Sugars released during digestion were determined as monosaccharides by the dinitrosalicylic

205 acid (DNS) colorimetric method after an invertase + amyloglucosidase secondary digestion

206 (Mishra, Monro, & Hedderley, 2008) with a small-scale modification. An aliquot of the

207 micellar phase (1 mL) was taken after 20 and 120 min of intestinal stage, mixed with absolute

208 ethanol (4 mL), and then centrifuged at 1000 x G during 10 min, at 20 °C. Afterwards, 0.05

209 mL of the supernatant, or glucose standard (1 mg/mL glucose) was mixed with 0.25 mL of an

210 enzymatic solution made of 1 % amyloglucosidase (E- AMGDF, Megazyme), 1 % invertase

211 (Sigma Aldrich) in acetate buffer pH 5.2 and 0.75 mL of the DNS solution (1 % glucose, 1 %

212 NaOH 1 M, 5 % DNS), and heated at 100 °C for 15 min. After heating, samples were cooled

213 and diluted with 4 mL of distilled water.

214   Starch measurement was recorded at 530 nm using a spectrophotometer (UV/vis, Beckman

215   Coulter). Rapid digested starch (RDS) was determined as reducing sugars measured after 20

216   min of intestinal stage while slowly digested starch (SDS) corresponded to the reducing sugars

217   measured after 120 min (RDS + SDS). Resistant starch (RS) was estimated by the difference

218   between RDS + SDS and total starch. Total starch was measured as glucose x 0.9 in a sample

219   of freeze-dried pasta that had been milled to a powder. A sub-sample (100 mg) was gelatinized

220   in dimethyl sulphoxide (2.0 mL, 100 ºC, 10 min) and digested for 30 min at 37 ºC after adding

221   8 mL acetate buffer pH 5.2 containing 0.1 mL of amyloglucosidase (Mishra et al., 2008).

222   *2.4.3. Total crocin and crocin isomers*

223   The content of crocin isomers was determined in agreement with the method reported in

224   Armellini et al. (2018). Crocins from freeze-dried pasta samples, micellar phases and saffron

225   powder (0.1 g) were extracted with methanol (5 mL, 80 % in water) using an Intell-Mixer RM-

226   2 (Elmi Ltd, Riga, Latvia). Samples were rotated head-over-heels at 55 rpm for 2 h at room

227   temperature and then centrifuged at 1200 x G for 10 minutes. The solid pellet was extracted

228   again following the same procedure. The supernatants of the two extractions were collected

229   and mixed (10 mL), and then they were used for HPLC analysis.

230   Crocetin esters were determined by HPLC and carried out by using a Waters instrument

231   comprising a pump and DAD detector (Waters, USA) equipped with a Kromasil C18 (100 A,

232   4.60 x 250 mm, 5 µm) column. The eluents were water (A) and acetonitrile (B) with the

233   following gradient: 95% to 5% A, 0-40 min; 5% A, 40-50 min; 5% to 95% A, 50-55 min at a

234   flow rate of 1 mL/min. 20 µL sample injections were made for all samples and standards. The

235   identification of crocin was carried out by comparing its retention time with that of the standard

236   at 440 nm; quantification was performed by external calibration curves (10, 20, 50, 100 and

237   150 mg/L), with six replicates for each level (n=6), being 0.002 mmol/L of crocin (Trans-4-

238   GG) the limit of quantification. Quantification of crocetin isomers was carried out referring to

239   the most abundant isomer, expressed as mg of crocin/g dry base.

240   The crocetin isomers were determined in cooked pasta (before digestion) and different

241   digestion times (120 min of gastric stage and 10, 20, 30, 45, 60, 90 and 120 min of intestinal

242   stage). One tube was used for each different sampling time.

243   In addition, bioaccessibility at the end of the intestinal stage (%) was calculated according to

244   Eq. (1) (Asensio-Grau et al., 2018):

245

246   Bioaccessibility (%)= A/B 100   (1)

247

248   where A is total crocin content (µg/g dry basis of pasta) determined in the supernatant at the

249   end of gastrointestinal digestion and B is total crocin in pasta before digestion and expressed

250   in the same units.

251

252   *2.5. Statistical analysis*

253   Results are reported as average ± standard deviation of nine measurements. Analysis of

254   Variance (Multivariate ANOVA) followed by Fisher LSD post-hoc tests was performed using

255   IBM SPSS Statistics 23 (IBM, USA), differences were considered statistically significant when

256   p-value < 0.05.

257

258   **3. Results and Discussion**

259   *3.1. Effect of saffron enrichment, oil addition and cooking time on Matrix Degradation*

260   *Index (MDI (%)) of digested fresh pasta*

261   During digestion, the absorption of a significant amount of water by the matrix, combined with

262   the action of digestive enzymes, promotes the softening of the food matrix and the reduction

11

263   of cohesive forces that hold the matrix together, resulting in the different degradation profiles

264   (Kong & Singh, 2009).

265   The matrix degradation index (MDI), corresponding to the amount (as %) of finely dispersed

266   pasta solids, was used as an estimation of mechanical disruption during the gastro-intestinal

267   digestion. **Figure 1** reports the values of the MDI after gastric and intestinal stages of control

268   and saffron-enriched pasta, with or without addition of oil. Results show that digestion time as

269   well as stage (gastric vs. intestinal) affected significantly the MDI.

270   Gastric stage (120 min) induced a MDI of ca. 55 % in the control sample after 1.5 minutes of

271   cooking (0%-1.5'). A significant increase of MDI, up to ca. 60 % (p-value < 0.05), was

272   observed in saffron enriched samples (0.2 or 0.4 %w/w), being more noticeable in the 0.4%-

273   enriched pasta cooked for 3 min (0.4%-3'). Furthermore, the addition of oil prior to digestion

274   (0.2+1.5'+oil 0.4+1.5'+oil), led to an increase of the MDI up to 65 %, compared to the control

275   (0%-1.5').

276   This degradation is the result of the activity of α-amylase present in the saliva that starts

277   breaking the starch branches into maltose and dextrin, followed by that of the gastric pepsin

278   which, in combination with the mechanical forces acting during the gastric stage, contributes

279   to break the food matrix. The degradation degree of pasta will depend on the characteristics

280   and composition of the food matrix. Saffron addition into the pasta did not have a significant

281   effect on the textural parameters of the raw pasta while a main effect was observed as a

282   consequence of cooking time. Food matrix became softer and less cohesive after 3 min of

283   cooking compared to that boiled for just 1.5 min (Armellini et al., 2018). Food matrix

284   characteristics such as hardness, cohesiveness, and elasticity have been previously associated

285   with resistance of other foods, such cheese, to the gastric environment (Lamothe et al., 2017).

286   Other factors, such as the composition of the matrix, the nature of bonds, and the permeability

287   of the matrix to small molecules could also influence matrix degradation (McClements,

288 Decker, Park, & Weiss, 2008). In addition, this resistance of the food matrix to degradation

289 might have an effect on the antioxidant release from the matrix into the digestion juices

290 (Lamothe et al., 2017).

291 During the intestinal stage, the entity of digestion significantly increased, reaching values

292 above 80 % after 120 min in all samples regardless saffron addition or cooking time. The

293 increase of MDI after the intestinal stage is the result of an enhanced enzymatic activity. The

294 action of the amylases, as well as the proteolytic enzymes (trypsin and chymotrypsin), present

295 in duodenal juices, contributed to the degradation of the gluten matrix (Lamothe et al., 2014).

296

297 **3.2. Effect of saffron enrichment, oil addition and cooking time on Starch digestibility of**

298 **fresh pasta**

299 Pasta samples' starch digestibility was evaluated by determining the amount of

300 monosaccharides released after the in vitro gastrointestinal digestion. An increase of glucose

301 concentration occurs along the digestion process, indicating that a progressive enzymatic

302 activity proceeds during the whole digestion process being this the result of the α-amylase in

303 saliva (oral stage), as well as pancreatic amylase activity (intestinal stage).

304 **Table 2** reports the values of the different fractions of starch (Rapidly Digested Starch (RDS),

305 Slowly Digested Starch (SDS), and Resistant Starch (RS)) for each pasta sample. The addition

306 of saffron to pasta (0.2%-1.5' and 0.4%-1.5') led to a slightly increase of SDS compared to the

307 control (0%-1.5') corresponding to an increase of 3.8 % of the total starch (TS). This increase

308 was statistically significant for the sample with the highest saffron concentration (0.4%-1.5')

309 in respect to the lower ones (i.e.3.8 % and 6.5 % of the TS respectively, for 0.2 and 0.4 % of

310 added saffron).

311 A slow digestion of starch contributes to limit the increase of the glycaemic index, which is

312 related to the blood glucose concentration after food consumption (Bohn et al., 2017). Based

313   on these results, it can be supposed that saffron enrichment might have a hindering effect on

314   starch degradation and glucose release, as it has been previously reported for other ingredients

315   added in pasta formulation (Heo et al., 2013).

316   By comparing the results obtained for pasta enriched with 0.4 % of saffron extract after 1.5 and

317   3 min of cooking, longer cooking time led to a significant decrease of the RDS fraction (19 %

318   *vs* 8 % for 1.5 and 3 min, respectively). A similar effect was noticed by Ye & Sui, (2016), who

319   studied the effect of cooking time on starch digestibility of Chinese noodles. In their study,

320   they concluded that cooking enlarged the voids around swelling starch granules and decreased

321   the amount of continuity of intervening proteins, leading to the greater accessibility of α-

322   amylase to the starch polymers (Fardet et al., 1998). The transformation of protein structure

323   could increase the accessibility of α-amylase to the swollen starch granules, resulting in an

324   increased starch digestion. In contrast, when noodles were conducted from an optimal cooked

325   state to an overcooked state, proteins were likely to refold and aggregate with inter- and intra-

326   molecular interactions, in turn containing strongly linked starch polymers leading to a lower

327   digestion effect (Zhang & Hamaker, 2009).

328   However, the mechanisms by which the gluten network slows digestion rates of entrapped

329   starch are not fully understood. The most common explanation is that the gluten network,

330   entrapping starch granules, acts as a barrier to inhibit the accessibility of enzymes. It may also

331   limit water absorption by starch granules, limiting the degree to which the starch is able to

332   swell and hence gelatinise during pasta cooking in excess water; this might limit as well the

333   ability of enzymes to access available starch and therefore, decrease the rate of starch digestion

334   (Colonna et al., 1990). Some authors have suggested that the low starch digestion rates of pasta

335   may be attributed to the tortuosity of the gluten network, which lengthens the pathway that α-

336   amylase must take to reach its substrate (Fardet et al., 1998).

Moreover, our results highlight that the addition of oil prior digestion seems to modify starch digestibility with a decrease in RDS fraction. Concretely, an increase of SDS was noticed for 0.2%-1.5'+oil and a significant increase of RS for 0.4%-1.5'+oil compared to the same samples without oil addition. The addition of a lipid phase seems to have an important impact on the conformation of starch fractions. Rodríguez-Huezo et al., (2018), analysed the effect of the addition of different animal and vegetal fats on Tamales, Mexican traditional maize-based food, formulation. In this study, X-ray and DSC analyses revealed the formation of stable starch-lipid complexes, which are structures with the ability of opposing the attack of digestive enzymes. Apparently, the formation of additional SDS observed in 0.2%-1.5'+oil sample and RS in 0.4%-1.5'+ oil sample were obtained at the expenses of rapid digestible starch (RDS) fraction. Therefore, it may be concluded that the oil addition may exert a hypoglycaemic effect.

3.3. Crocin, crocin isomers and their bioaccessibility after digestion

The commercial saffron used for the present study to enrich pasta was characterized in a previous study (Armellini et al., 2018) and classified as II category (ISO 3632-1:2011), with the following quality parameters: colour (A 1% 1cm, 440 nm)=169; aroma (A 1% 1cm, 330 nm)=41; flavour (A 1% 1cm, 257 nm)=67 and a moisture content (%)=11.86.

The main cis- and trans-crocetin esters were identified at 440 nm: trans-crocetin di-(b-D-gentiobiosyl) ester (trans-4-GG), was identified as crocin by comparison with its standard, as well as with literature; the other isomers tentatively identified were: trans-crocetin (b-D-glucosyl)- (b-D-gentiobiosyl) ester (trans-3-Gg); trans-crocetin di-(b-D-glucosyl) ester (trans-2-gg); cis-crocetin di-(b-D-gentiobiosyl) ester (cis-4-GG); cis- crocetin (b-D-neapolitanosyl)-(b-D-glucosyl) ester (cis-4-ng), and cis-crocetin (b-D-glucosyl)-(b-D-gentiobiosyl) ester (cis-3-Gg) according to the literature (Armellini et al., 2018; García-Rodríguez et al., 2017).

Table 3 shows the concentration of total crocin (µg/g pasta (d.b.)) and *trans/cis*-isomers

362   distribution (%) of saffron-enriched pasta before digestion, after gastric stage (GS) and at

363   different times of the intestinal stage (IS).

364   Crocin content in samples (before digestion) was not only dependant on saffron addition (0.2

365   or 0.4%), but also on cooking time and oil presence during dish preparation. Of note, the higher

366   whichever of three factors, the higher the total crocin content in pasta (0.4%-3' ≈ 0.4%-1.5'+oil

367   > 0.4%-1.5'+oil ≈ 0.4%-1.5' > 0.2%-1.5').

368   It could be observed that cooking and oil addition enhanced solubility of crocin from the food

369   matrix during solvent extraction leading therefore, to a higher content quantified by

370   chromatography. Isomerization trans-cis was also highly affected by cooking as it can be

371   noticed from the results obtained for 0.4'-3% pasta, which presented the higher cis-isomers

372   percentage (53 %) compared to the samples cooked for a shorter time, even when oil was

373   added. It should be pointed out, that *trans-4-GG* and *cis*-3-Gg were, in general, the most

374   abundant isomers before and after digestion regardless pasta formulation or trans-cis isomers

375   distribution (%).

376   Although safranal was identified in the different samples, its concentration was below the

377   LOQ, and therefore the amount in samples could not be quantified.

378   During gastrointestinal simulation, total crocin was clearly affected by the concentration of

379   saffron extract added to the pasta dough (0.2 vs 0.4 %). The higher the saffron powder

380   concentration, the higher the amount of crocin released into the digestion fluids at both, gastric

381   and intestinal, stages. As regards the cooking time, the sample cooked for a longer time (0.4%-

382   3') showed a lower release of crocins into the digestion fluids despite of its highest crocin

383   content before digestion. This is in agreement with the results of the starch digestibility already

384   discussed, where inter- and intra-molecular interactions resulting from the overcooking of

385   pasta, led to a lower digestion, and, thus, to a lower bioactives release from the food matrix

386   (Lamothe et al., 2017; Ye & Sui, 2016). Finally, the addition of oil to pasta after cooking

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seemed to significantly influence crocin release during digestion, and especially during the

intestinal stage due to the liposoluble nature of this compound. To be absorbable in the human

body, carotenoids need to be released from the food matrix and dispersed into the lipid phase

in the stomach, where a fine emulsion structure is created. In a next step, the carotenoids need

to be incorporated into mixed micelles in the intestine (Pasqualone et al., 2016). The presence

of lipids in the food/dish preparation are essential for the emulsification and micellation. The

initiated *trans* to *cis* isomerization in samples progressed during digestion as well; and it was

also enhanced by both cooking time (0.4%-3') and oil addition (0.2%-1.5'+oil and 0.4%-

1.5'+oil), as it can be deduced by comparing the *cis*-isomers (%) in these samples compared to

that value in 0.2%-1.5' and 0.4%-1.5'. Nevertheless, an increase of saffron powder in the dough

pasta from 0.2 to 0.4% (w/w), without an increase of cooking time, implied an increase of total

crocin content but not an activation of *trans-cis* isomerization during digestion. In fact, *trans*-

isomers represented more than 80 % of total crocin in 0.2%-1.5 and 0.4%-1.5' at 120 min of

the intestinal stage.

Bioaccessibility (%) of each pasta estimated as total crocin content at the end of gastrointestinal

digestion with respect of total crocin in pasta before digestion, was equal to 2.9 ±1.1, 73 ± 10,

25 ± 7, 62 ± 9 and 97 ± 3 % in 0.2%-1.5', 0.4%-1.5', 0.4%-3, 0.2%-1.5'+oil and 0.4%-1.5'+oil,

respectively.

Based on the results of the crocins content the enrichment of pasta with saffron extracts

especially when the highest concentration of extract (0.4%) is used and pasta overcooked (3

min) may lead to obtain levels of crocins (ca. 1g/100 g pasta) that may have potential toxic

effects or adverse effects on humans. Over the decades, several studies have focused on the

toxic effects of saffron, which might include vomiting, uterine bleeding, bloody diarrhoea,

haematuria, bleeding from the nose, lips and eyelids, vertigo, numbness and yellowing of the

skin and mucous membranes. However, most of those reports are old and the respective

17

412 amounts have been in times questioned (Lymperopoulou & Lamari, 2015; Smith & Zeeman,

413 2006). Some studies report that oral administration of doses up to 3 g crocin /kg of weight,

414 within 2 days in mice did not cause mortality, and similar results were observed after intra

415 peritoneal (IP) exposure at the same dose (Bostan, Mehri, & Hosseinzadeh, 2017;

416 Hosseinzadeh, Sadeghi Shakib, Khadem Sameni, & Taghiabadi, 2011)). Furthermore, different

417 studies have proved crocin to be a practically low-toxic substance, which according to the

418 toxicity classification, corresponds to a LD50 (lethal dose) value within the range of 1–5 g/kg

419 (Hosseinzadeh, Sadeghi Shakib, Khadem Sameni, & Taghiabadi, 2011; Kennedy, Ferenz, &

420 Burgess, 1986). The consumption of saffron enriched pasta, even in the case of the product

421 with the highest content of crocins will, thus, not represent a risk.

422 **Figure 3** shows the evolution of crocetin isomers in the digestion fluids during the intestinal

423 stage of the in vitro digestion process for the saffron-enriched pasta samples. Intestinal

424 conditions led to an increase *trans-cis* isomerization, being the final concentration dependent

425 on cooking time and added oil. Among the identified *trans* and *cis*-isomers, *trans*-4-GG and

426 *cis*-3-Gg presented the highest concentrations, respectively.

427 Additionally, *trans*-crocin isomers concentration (*trans*-4-GG, *trans*-3-GG, *trans*-2-gg)

428 reached a maximum concentration after 20-30 min of the intestinal stage, and it decreased from

429 this time onwards. An exception of the trend was observed in overcooked pasta (0.4%-3'), for

430 which *trans* isomers concentration continuously augmented with respect to intestinal time. The

431 observed decrease of *trans* isomers concentration after 30 min of intestinal digestion might be

432 probably due to a pH change in the environment. In fact, according to Pineda-Vadillo et al.,

433 (2017), the change from the acidic gastric media to the mild alkaline intestinal environment,

434 could induce physico-chemical modifications of molecules that influence their transfer to

435 digestion fluids (solubility), and motivate degradation reactions along with the isomerization

436 mechanism *trans-cis*. The same phenomenon has been reported for tomato-lycopene. García-

18

437 Hernández, et al., (2018) reported a *trans-cis* isomerization during frying and during the further

438 gastrointestinal digestion, being the final distribution of lycopene in micelles close to 50 %.

439 Similarly, *cis*-isomers of lycopene have been detected in blood samples reaching 60-80 % of

440 total lycopene even though lycopene occurs in food mainly in the *trans* form (Wu et al., 2003).

441 Degradation reactions during digestive process have been also reported to occur also for other

442 antioxidant molecules, such as polyphenols. A decrease in the amount of total flavonoids and

443 total phenols in the bioaccessible fraction, was found due to the conversion of flavanones into

444 chalcones, which are less soluble than flavones, and therefore less available for absorption (Gil-

445 Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001). In addition to pH, the interaction between

446 polyphenols and other components of the in vitro gastro-intestinal digestion like enzymes or

447 other dietary components released during digestion (e.g. iron, other minerals, dietary fiber,

448 proteins) might affect polyphenols solubility and bioaccessibility (Rodríguez-Roque et al.,

449 2015). The same phenomenon might, thus, occur to crocin, negatively affecting its

450 bioaccessibility.

451 On the other hand, the higher the amount of added saffron, the higher content of crocetin

452 isomers in the digestive juices was observed with a significant effect of oil addition on isomers

453 release, and particularly that of the *cis*-3-Gg isomer. These results highlight the affinity of the

454 different isomers for the lipidic phase depending on their molecular structure, since some of

455 them were detected in higher amounts than others in the digested fluids when oil was added. A

456 number of studies have been conducted on the effect of the presence of oil on carotenoids

457 bioaccessibility. Most of them report the enhancement of carotenoids bioaccessibility in fruit-

458 and vegetable-based food products, when olive oil, or to a minor extent sunflower oil and

459 soybean oil, are added. Olive oil contains mainly oleic acid, a monounsaturated long chain fatty

460 acid (C18:1). The fatty acyl chain length has been identified as an important lipid feature with

461 consequence on carotenoid bioaccessibility.

462 Eventually, the molecular size and structure of carotenoids, which are related to isomeric

463 configuration, also seem to influence bioaccessibility (Nagao, Kotake-Nara, & Hase, 2013).

464

465 **4. Conclusions**

466 The effect of saffron extract addition on fresh pasta in-vitro starch digestibility and the fate and

467 release of bioactives and, in particular, crocins during digestion has been investigated. Results

468 evidenced that saffron extract seems to effect starch digestibility, with a likely decrease of

469 starch digestibility and corresponding glycaemic index of the saffron-enriched pasta.

470 Concentration of saffron extract as well as cooking time significantly influenced crocin release

471 in the digested fluids. The higher the saffron powder concentration and the shorter the cooking

472 time, the higher is the amount of crocins released into the digestion fluids. Moreover, the

473 addition of oil to the pasta prior to digestion significantly enhanced crocins release into the

474 micellar phase during intestinal stage. Additionally, the isomerization from *trans* to *cis*

475 occurred mainly during digestion, in the 3 min-cooked enriched saffron pasta likely favoured

476 again by the presence of a lipidic dispersed phase that lead to a higher concentration of the *cis*

477 isomers in the bioaccessible fraction of this system.

478 This preliminary study on the use of saffron as ingredient in formulated pasta products and its

479 results could be a reference to be exploited in further investigations aimed to deepen the

480 knowledge and applications of this spice and related bioactive compounds, their technological

481 functionality, and their impact on food quality as well as human health and wellbeing.

482

483 **Conflicts of interest**

484 There are no conflicts of interest to declare.

485

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624
Table 1. Composition of simulated digestion fluids

Constituent	SSF	SGF	SIF
	$mmol \cdot L^{-1}$	$mmol \cdot L^{-1}$	$mmol \cdot L^{-1}$
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2$	1.5	0.15	0.6

625 The addition of pepsin, Ca^{2+} solution and water will result in the correct electrolyte concentration in the final

626 digestion mixture.

627

628 **Table 2**. Starch fractions of digested saffron-enriched fresh pasta (0, 0.2 or 0.4 % of saffron

629 (w/w)) cooked at different times (1.5 and 3 minutes) and with or without oil addition. RDS:

630 Rapidly Digested Starch; SDS: Slowly Digested Starch; RS: Resistant Starch are expressed as

631 percentage (%) on total starch. Samples are codified as follows: saffron content in dough pasta

632 (0.2 or 0.4 %)-cooking time (1.5 or 3 min) + oil addition.

	RDS(%)	SDS(%)	RS(%)
0%-1.5' (control)	21.9 ± 1.3^d	4.00 ± 0.05^a	73.8 ± 2.3^a
0.2%-1.5'	17.4 ± 1.8^c	4.6 ± 1.5^{ab}	79.1 ± 0.8^b
0.4%-1.5'	26 ± 5^d	5.7 ± 0.6^b	75.0 ± 4.5^a
0.4%-3'	8.9 ± 0.2^a	5.4 ± 0.2^b	86.2 ± 2.4^c
0.2%-1.5' + oil	14.5 ± 0.2^b	10.0 ± 1.7^c	75.6 ± 1.9^a
0.4%-1.5'+ oil	14.22 ± 0.05^b	4.27 ± 0.07^c	85.1 ± 4.8^c

633   [a-d] Different letters indicate the homogeneous groups obtained by the

634   analysis of variance (ANOVA p-value < 0.05)

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636

637

638 **Table 3.** Total crocin content (µg/ g pasta (dry base) and *trans* and *cis*-isomers distribution (%)

639 in saffron-enriched pasta before and during the gastrointestinal digestion (GS: Gastric Stage;

640 IS: Intestinal Stage). Samples are codified as follows: saffron content in dough pasta (0.2 or

641 0.4 %)-cooking time (1.5 or 3 min) + oil addition.

|		Total crocin (µg/g pasta (d.b.))	*Trans*-isomers (%)	*Cis*-isomers (%)
0.2%-1.5'	**Pasta**	3549.6 ± 0.3^{aE}	80.772 ± 0.003^{dA}	19.23 ± 0.02^{dA}
	GS-120 min	625 ± 188^{aCD}	93.5 ± 0.5^{cC}	6.5 ± 0.5^{cC}
	IS-10 min	773 ± 114^{aD}	93.5 ± 0.7^{eC}	5.4 ± 0.7^{eC}
	IS-20 min	630 ± 150^{aCD}	99.03 ± 0.07^{dD}	0.97 ± 0.07^{dD}
	IS-30 min	429 ± 98^{aBC}	96 ± 2^{dCD}	4 ± 2^{dCD}
	IS-60 min	188 ± 54^{aAB}	96 ± 2^{dC}	5 ± 2^{dC}
	IS-90 min	322 ± 97^{aAB}	95.4 ± 0.2^{dC}	4.6 ± 0.2^{dC}
	IS-120 min	102 ± 39^{aA}	88 ± 2^{dB}	12 ± 2^{dB}
0.4%-1.5'	**Pasta**	4236 ± 13^{bCD}	99.7 ± 0.3^{eE}	0.3 ± 0.3^{eE}
	GS-120 min	1824 ± 290^{bABC}	59.0 ± 1.3^{aA}	41.0 ± 1.3^{aA}
	IS-10 min	1846 ± 216^{bA}	87.169 ± 1.105^{dCD}	12.831 ± 1.105^{dCD}
	IS- 20 min	4901 ± 1405^{bD}	90.80 ± 1.12^{cD}	9.20 ± 1.12^{cD}
	IS- 30 min	3194 ± 143^{cABC}	86 ± 3^{cBC}	14 ± 3^{cBC}
	IS- 60 min	2663 ± 412^{bAB}	87 ± 4^{dCD}	13 ± 4^{dCD}
	IS- 90 min	3562 ± 749^{bBCD}	88 ± 3^{dCD}	12 ± 3^{dCD}
	IS- 120 min	3099 ± 431^{bABC}	80.8 ± 0.6^{cB}	19.2 ± 0.6^{cB}
0.4%-3'	**Pasta**	10483.1 ± 0.8^{eE}	46.961 ± 0.002^{aA}	53.039 ± 0.002^{aA}
	GS- 120 min	1183 ± 427^{aA}	92.6 ± 0.9^{cF}	7.4 ± 0.9^{cF}
	IS- 10 min	1738 ± 208^{bABC}	80.8 ± 0.6^{bE}	19.2 ± 0.6^{bE}
	IS- 20 min	3985 ± 620^{bD}	53.6 ± 2.7^{aB}	46.4 ± 2.7^{aB}
	IS- 30 min	1444 ± 79^{bAB}	89.9 ± 0.7^{cF}	10.1 ± 0.7^{cF}
	IS- 60 min	2322 ± 920^{bABC}	53.71 ± 0.06^{bBC}	46.29 ± 0.06^{bBC}
	IS- 90 min	2724 ± 542^{bC}	58 ± 3^{bD}	42 ± 3^{bB}
	IS- 120 min	2618 ± 742^{bBC}	57 ± 2^{bCD}	43 ± 2^{bCD}
0.2%-1.5'+oil	**Pasta**	4341 ± 40^{cD}	60.6 ± 0.6^{bB}	39.4 ± 0.6^{bB}
	GS- 120 min	919 ± 36^{aA}	95 ± 2^{cE}	5 ± 2^{cE}
	IS- 10 min	1880 ± 358^{bB}	83 ± 0.2^{cD}	16.7 ± 0.2^{cD}
	IS- 20 min	3901 ± 190^{bD}	53.4 ± 1.3^{aB}	46.6 ± 1.3^{aB}
	IS- 30 min	1965 ± 109^{bBC}	42 ± 2^{aA}	58.2 ± 2.3^{aA}
	IS- 60 min	1838 ± 427^{bB}	43 ± 6^{aA}	57 ± 3^{aA}
	IS- 90 min	2153 ± 601^{bBC}	73 ± 7^{cC}	28 ± 7^{cC}
	IS- 120 min	2675 ± 408^{bC}	57 ± 2^{bB}	43 ± 2^{bB}
0.4%-1.5'+oil	**Pasta**	9420 ± 3^{dEF}	73.470 ± 0.007^{cD}	26.530 ± 0.007^{cD}
	GS- 120 min	3016 ± 259^{bA}	84.4 ± 0.7^{bE}	15.6 ± 0.7^{bE}
	IS- 10 min	7094 ± 339^{cD}	76 ± 2^{aD}	24 ± 2^{aD}
	IS- 20 min	5006 ± 186^{bB}	86 ± 2^{bE}	14 ± 2^{bE}
	IS- 30 min	5973 ± 632^{dC}	75 ± 2^{bD}	25 ± 2^{bD}
	IS- 60 min	4861 ± 469^{cB}	65 ± 3^{cC}	35 ± 3^{cC}
	IS- 90 min	10190 ± 687^{cF}	29.67 ± 1.04^{aA}	70.33 ± 1.04^{aA}
	IS- 120 min	9118 ± 279^{cE}	34.4 ± 0.5^{aB}	65.6 ± 0.5^{aB}

642 [a-d] Different letters refer to the homogenous groups obtained for different pasta formulations (0.2%-1.5', 0.4%-1.5', 0.4%-

643 3', 0.2%-1.5'+oil, 0.4%-1.5'+oil) at a statistical significance of 95% (p-value < 0.05). [A-F] Different letters refer to the

644 homogenous groups obtained after digestion (pasta) and for the different times of digestion (GS-120 min, IS-10min, IS-20

645 min, IS-30 min, IS-60 min, IS-90 min and IS-120 min) at a statistical significance of 95% (p-value < 0.05).

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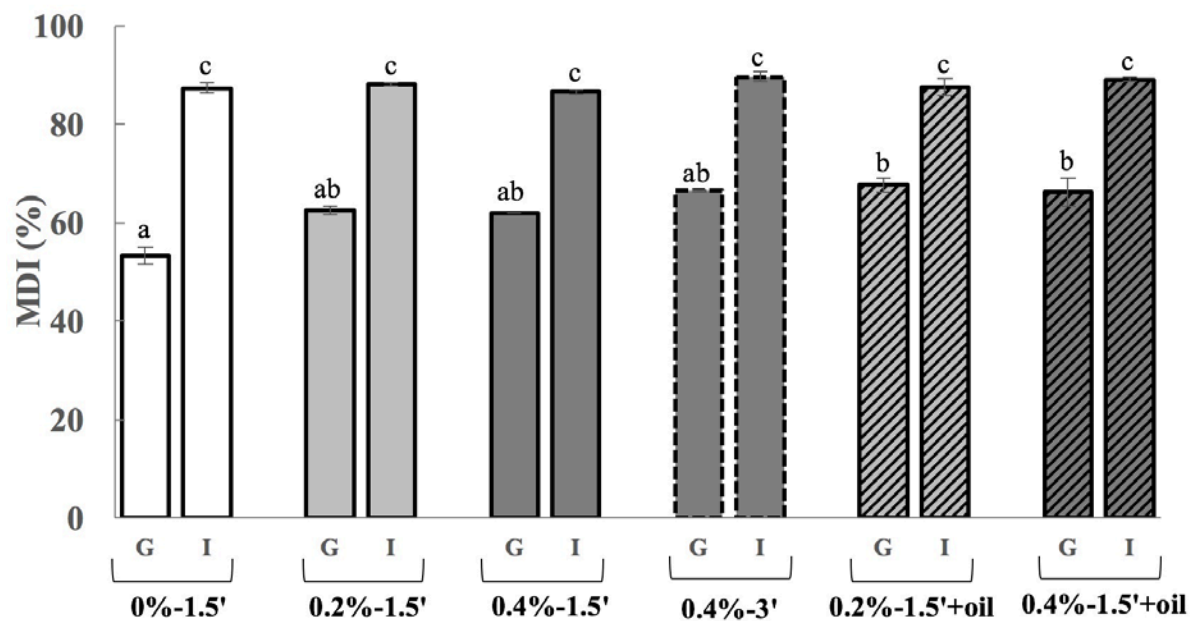
Figure 1. Matrix degradation index (MDI (%)) achieved after the in vitro gastrointestinal digestion of saffron-enriched pasta with or without oil addition. Samples are codified as follows: saffron content in dough pasta (0.2 or 0.4 %)-cooking time (1.5 or 3 min) + oil addition.

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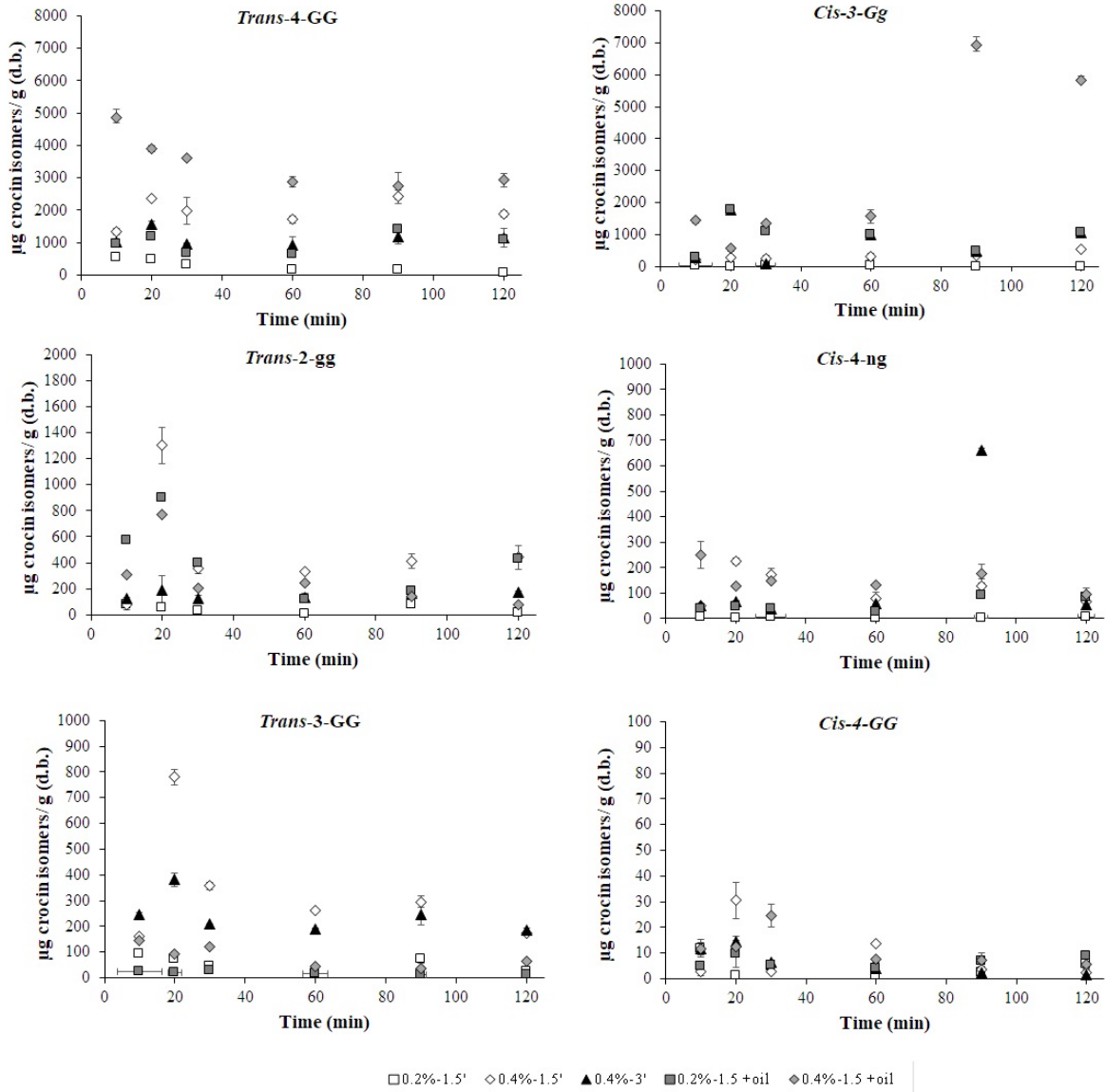
Figure 2. Concentration of the principal crocetin isomers (mg/g d.b.) identified by HPLC in

the different saffron-enriched pasta at different interval times of intestinal digestion. Samples

are codified as follows: saffron content in dough pasta (0.2 or 0.4 %)-cooking time (1.5 or 3

min) + oil addition.

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