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Mitigation Strategies to Reduce Acrylamide in Cookies: Effect of Formulation

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Maria Alessia Schouten; Silvia Tappi; Pietro Rocculi; Santina Romani

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1 **Mitigation Strategies to Reduce Acrylamide in Cookies: Effect of**
2 **Formulation**

3 Maria Alessia Schouten^{a,*}, Silvia Tappi^{a,b}, Pietro Rocculi^{a,b}, Santina Romani^{a,b,*}

4

5 *^aDepartment of Agricultural and Food Sciences, Alma Mater Studiorum, University of*
6 *Bologna, Campus of Food Science, Piazza Goidanich 60, 47023 Cesena (FC), Italy;*

7 *^bInterdepartmental Centre for Agri-Food Industrial Research, Alma Mater Studiorum,*
8 *University of Bologna, Via Quinto Bucci 336, 47023 Cesena (FC), Italy*

9

10 **ORCID:** Maria Alessia Schouten (0000-0001-7182-8559); Silvia Tappi (0000-0003-0711-
11 859X); Pietro Rocculi (0000-0002-1255-3185); Santina Romani (0000-0002-5586-8443).

12

13 ***Corresponding Authors.** E-mail address: maria.schouten2@unibo.it (M.A. Schouten);
14 Santina.romani2@unibo.it (S. Romani). Postal address: Department of Agricultural and Food
15 Sciences, Alma Mater Studiorum, University of Bologna, Campus of Food Science, Piazza
16 Goidanich 60, 47023 Cesena (FC), Italy (M.A. Schouten; S. Romani).

17

18 **Mitigation Strategies to Reduce Acrylamide in Cookies: Effect of**
19 **Formulation**

20 Acrylamide (AA) is a well-known toxic compound formed in various foods during the
21 high thermal process. Cookies, one of the most consumed bakery goods worldwide,
22 represent a category of food at risk of AA in the human diet. Therefore, some strategies
23 for its control in cookies should be employed. The present review summarizes and
24 discusses the mitigation strategies for AA reduction, reported in scientific literature,
25 that could be carried out during the cookie's formulation and some of their effects on
26 the final product quality. The evaluation of AA formation related to various ingredients
27 could help the food industries and researchers to develop a more effective method to
28 reduce this toxic compound in cookies, as well as in other bakery products.

29 Keywords: Acrylamide mitigation; Processing contaminant; Cookie; Formulation;
30 Bakery ingredients.

31 **1. Introduction**

32 Bakery products such as bread, breakfast cereals, crackers, wafers, and cookies represent a
33 key part of the human diet. Cookies are one of the most appreciated and consumed bakery
34 goods worldwide, thanks to their ready-to-eat nature, availability in numerous varieties, long
35 shelf-life, and relatively low cost. ^[1-3] However, cookies greatly contribute to the dietary
36 acrylamide (AA) intake, especially in infants (<2 years of age) and children (>2 years of age)
37 with contributions of about 27 and 56%, respectively. ^[4]

38 AA is a toxic and carcinogenic compound naturally formed during baking and other
39 food processing/cooking methods performed at high-temperature above 120 °C. ^[5-7] The
40 toxicological effects of AA on humans are neurotoxicity, genotoxicity, carcinogenicity, and
41 reproductive toxicity, leading to its classification as a Group 2A carcinogen by the
42 International Agency for Research on Cancer. ^[8] After its absorption from the gastrointestinal
43 tract, AA is metabolized to glycidamide, a mutagenic and genotoxic compound, following
44 the reaction catalyzed by the cytochrome enzyme. ^[6, 9, 10] Glycidamide formation is
45 considered responsible for the genotoxic effects of AA having the potential to induce
46 mutagenic genes at the chromosomal level. ^[10]

47 The formation of AA in cookies is derived mainly from the Maillard reaction and is
48 firstly related to the ingredient types and quantity used in the formulation, as well as their
49 interactions during the entire preparation process which also includes the use of high
50 temperatures during baking. The main precursors of AA are reducing sugars and free
51 asparagine; as sugars are normally abundant in dough formulations, the concentration of free
52 asparagine is the rate-limiting factor for AA formation in cookies. ^[11, 12] Extensive scientific
53 efforts have been carried out aiming at mitigation and control of AA formation in cookies by
54 the modification and optimization of the dough formulations. A total of 62 research papers

55 are present in the scientific literature since 2004, as showed in Figure 1a. No research works
56 were published in 2005. The number of original articles has grown exponentially with a high
57 number of new studies especially in 2012, 2019, and 2020, probably as a result of the
58 introduction of different legal regulations, AA reference levels, and guidelines.

59 Considering the presence of AA in foods and its health risk, several recommendations
60 and regulations have been established by the European Commission over the years. Among
61 the most relevant, after several annual monitoring of AA levels in different food products
62 and in agreement with the European Food Safety Authority (EFSA) opinion, there is the
63 European Commission recommendation (EC 10.1.2011), that introduced the concept of AA
64 indicative values in foods. ^[13] These AA indicative values were not intended as regulatory
65 limits or safety thresholds but were set for different food categories at levels that the food
66 industry should be able to achieve based on EFSA's 2007-2008 monitoring data. ^[13]
67 Subsequently, the EC Regulation (EU 2017/2158) was introduced, establishing mitigation
68 measures and reference levels for the reduction of the presence of AA in three food categories
69 including coffee, potatoes, and bakery products. According to this Regulation, the food
70 business operators are obliged to apply measures to reduce the level of AA in order to reach
71 the lowest possible level below the reference one established in this normative act.
72 Concerning the category “cookies and wafers”, the AA benchmark value is 350 µg/kg. ^[14] In
73 addition, because it was concluded that this regulation did not present sufficient available
74 data on the presence of AA in foods, a more recent European Commission Recommendation
75 2019/1888/EC introduced a new list of non-exhaustive food products, including some bakery
76 products specialties (e.g., buns, sticks, pancakes, etc.), which must be monitored to identify
77 the AA risk and adopt new prevention and/or reduction measures against this food
78 contaminant. ^[10, 15]

79 The selection of conventional ingredients such as flours, sugars, leavening agents,
80 salts, oils, fats and additional ones including organic acids, amino acids, enzymes and
81 antioxidants, can control the presence of AA precursors and reduce its formation in cookie
82 products. The type of ingredients investigated in the reported research papers varied in
83 percentages greatly from year to year as shown in Figure 1b and in some cases, different
84 ingredients have been studied in the same work. The most studied ingredients for AA
85 mitigation in cookies were sugars, flour, and leavening agents. However, it must be taken
86 into consideration that any change aimed at improving cookie formulation can significantly
87 influence their overall quality. ^[16]

88 The present review article aims to thoroughly describe and discuss the mitigation
89 strategies of AA reported in the scientific literature, carried out in particular during the
90 cookie's formulation step, and some of their effects on the final product quality. In fact, the
91 evaluation of AA formation related to various ingredients used for cookie production could
92 help the food industries to develop more effective methods to reduce this toxic compound in
93 the final product as well as in other bakery products.

94

95 **2. Cookie formulation**

96 The bakery industry is in constant innovation, and bakery products are widely consumed
97 worldwide by different consumer groups. Cookies represent a very broad category of bakery
98 products and can be classified into different types based on their formulation: hard dough,
99 characterized by 6-10% of fat and 10-15% of sugar; short dough with low sugar (14-20%)
100 and low fat (12-19%); short dough with high sugar (15-30%) and high fat (18-22%); soft
101 dough with fat and sugar around 30% and 33-60% respectively. ^[17, 18] Hard dough cookies
102 are generally crispy and crunchy with an open texture (e.g., “tea cookies”, “garibaldi fruit”,

103 etc.), short dough ones are brittle and poorly plastic (e.g., shortbread, “Italian frollini”, etc.),
104 while soft dough ones are identified by a soft texture that makes them fragile and subjected
105 to breakage (e.g., sponge cookies, meringues, etc.).^[17, 19]

106 The ingredients normally used in the manufacture of cookies are wheat flour, sugar,
107 fat, water, eggs, and leavening agents in different proportions, depending on product type.
108 ^[19] Moreover, other ingredients can be incorporated or replaced in the formulation to obtain
109 different, innovative, and healthy cookie types such as pseudo-cereals (e.g., quinoa,
110 amaranth, buckwheat) or legumes flours (e.g., chickpea, lupine, soya), fat- and sugar-mimetic
111 ingredients (e.g., maltodextrin, lecithin, xylitol), antioxidant compounds (e.g., plant powders
112 and extracts) and flavors (e.g., chocolate, creams, nuts). The nature and quantity of
113 ingredients determine the sensorial and nutritional quality of the cookies.^[1]

114 Nearly all cookies are formulated with wheat flour as the most important and basic
115 constituent.^[20] The major functions of wheat flour in cookie dough are: to form the dough
116 during mixing, to hold all the ingredients uniformly distributed in the dough and making easy
117 machinability; to retain gas during mixing and baking; to form the structure of the product.
118 ^[21] Sugar is another important ingredient in cookies formulation that can vary from simple
119 sugars such as glucose and fructose to more complex ones such as sucrose and maltose. The
120 main sugars used in cookies preparation are sucrose in solid form, inverted syrup, glucose
121 syrup, honey, and high fructose products. The principal functions of sugar in the manufacture
122 of cookies are: to give a sweetish taste and flavor; to help water retention improving their
123 shelf life; to participate in caramelization and Maillard's reactions necessary for the formation
124 of aromas and color, and to give the right volume to the dough.^[21] Fats and oils are present
125 in cookies as dough ingredients, in surface sprays, cream fillings, coatings, or as a part of
126 other ingredients such as egg yolks and chocolate. Among them, vegetable oils, butter, and

127 shortenings are commonly used. The main functions of fats and oils in the formula of cookies
128 are: to give a tensor effect to the dough; to improve the machine workability of the dough; to
129 improve the palatability of the product. ^[21] As cookies are generally long-life products, any
130 used oil and fat must be stable under storage conditions. For this reason, antioxidants
131 ingredients are often added to the formulation to prevent oxidative rancidity and unpleasant
132 flavors. ^[21, 22] Water, together with other alternative liquid ingredients such as eggs and milk,
133 also plays an important role in the cookie's formulation. Water is necessary during the mixing
134 step, but it should be considered more correctly as a processing aid rather than an ingredient
135 because the water added as it is or through other ingredients is largely eliminated during the
136 baking process. Some water functions are: to help gluten formation and starch-swelling
137 processes; to bring dough ingredients in contact; to dissolve and distribute salt, sugars,
138 chemicals, and other water-soluble ingredients; to promote the enzyme activity; to assist
139 temperature control of dough and to help cookie aeration by the formation of steam during
140 baking. ^[21] According to some traditional recipes, in many types of cookies fresh eggs instead
141 of water are used. In addition to their high nutritional value, eggs are added for their
142 emulsifying, binding and yellow coloring functions particularly appreciated by the consumer.
143 ^[17] However, in some cookie types, fresh eggs are replaced by powdered eggs, which are
144 easier to use but with lower technological performances, or by fat and emulsifier substances
145 obtained from alternative sources. ^[17, 21] Concerning the leavening agents, cookies are usually
146 chemically leavened using different bicarbonates (sodium bicarbonate, NaHCO_3 , and
147 ammonium bicarbonate, NH_4CO_3). Chemical agents aerate the dough with the production of
148 carbon dioxide obtained by bicarbonates decomposition thanks to the high temperatures of
149 baking and through the chemical reaction of alkaline ingredients, such as bicarbonates, with
150 acidic ingredients ^[19], that are usually cream of tartar ($\text{KHC}_4\text{H}_4\text{O}_6$) or sodium aluminum

151 sulfate ($\text{NaAl}(\text{SO}_4)_2$).^[22, 23] The choice and the combination of different bicarbonates with
152 different acids result in the release of leavening gases with several profiles, that expand the
153 dough and impart specific sensorial characteristics to the final product.^[19, 24]

154 After the selection and dosing of the ingredients, also the other cookies process steps,
155 such as mixing, dough sheeting, dough sheet relaxation, shape forming, baking, cooling, and
156 packaging are important in determining the overall characteristics of the final different
157 product types.^[22, 25]

158

159 **3. Effect of cookies formulation on acrylamide formation**

160 A significant number of mitigation strategies to reduce AA content in food have been
161 proposed and tested so far. The first control step to reduce AA levels includes changes in
162 formulation, i.e. selection of raw materials and recipe of food products.^[26] The addition or
163 removal of some conventional ingredients of bakery products, such as flours, sugars,
164 leavening agents, salts, oils, and fats, or minor ingredients such as organic acids, amino acids,
165 enzymes, and antioxidants could potentially increase or decrease AA levels. In fact, due to
166 the mechanism of AA formation, the type and amount of all these ingredients can influence
167 the presence of precursors and/or the extent of the Maillard reaction.

168 ***3.1 Conventional ingredients***

169 ***3.1.1 Flours***

170 Flour is the basic ingredient that represents the highest concentration in the composition of
171 cookies and other bakery products. In general, each flour type, according to genetic basis,
172 growing conditions, agronomic factors, and post-harvest processing, has a different chemical
173 composition and physical properties.^[12, 27] Depending on the origin of the flour, this is the

174 main source of asparagine in the cookie formulation. Hence, it is important to understand
175 what factors influence its concentration in this ingredient and, consequently, the formation
176 of AA in baked products. Wheat flour is the most widely used; however recently, alternative
177 ones (i.e., not-wheat, not-cereals, pseudo-cereals, legumes) are increasingly used to improve
178 the nutritional value of bakery products. In addition to the given main nutrients and dough
179 technological properties, it is also necessary to determine their impact on the formation of
180 process contaminants such as AA. [28] Several authors have studied the impact on AA
181 formation of flour origin, mix of different flours, and their amount used in the cookie recipe,
182 as reported in Table 1.

183 Miśkiewicz et al. [29] studied the AA content in shortcrust cookies formulated with
184 wheat Poznań flour (type 500), spelt-wheat flour (type 630), and wheat Poznań flour blended
185 with flours from rice, chickpea, and amaranth seeds in the portions of 50, 50 and 25%,
186 respectively. The concentration of AA resulted of 41.9 µg/kg in the cookies based on wheat
187 flour only, while the samples prepared with a blend of wheat and chickpea flours resulted in
188 the lowest AA formation (5.7 µg/kg). These results were attributed to the lower sucrose and
189 reducing sugars content found in the blend of wheat and chickpea flours compared to wheat
190 flour only. In addition, the relatively low concentration of AA in the cookies produced from
191 this flour mixture could also result from the protective effect of chickpea proteins, which
192 limit the reactivity of AA precursors present in the raw dough during baking as previously
193 observed in fried potato products. [30]

194 Nowadays, soybeans are extensively consumed worldwide because of their several
195 technological and health benefits; Palermo et al. [31] proposed to evaluate the effect of freeze-
196 dried okara, a by-product of soybeans processing, on AA formation in cookies, obtained by
197 replacing 15% of wheat flour with this product. Cookie samples enriched with okara showed

198 a more intense development of the Maillard reaction leading to a higher formation of AA
199 (+60%) than in the control. According to the authors, this phenomenon could be linked to the
200 presence of about 50% insoluble dietary fiber in okara, which reduces the water activity of
201 dough during baking, thus favoring the Maillard reaction.

202 Another study of Mesías et al. ^[32] investigated the effect of replacing up to 20% of
203 wheat flour with chia seeds flour in the cookie dough on the nutritional properties, antioxidant
204 content, and the formation of food toxic compounds, including AA, of the final product, with
205 the purpose to evaluate the risk/benefit of the new formulations. The incorporation of chia
206 flour into the formulation of wheat-based cookies resulted in a nutritionally enhanced product
207 with a higher amount of protein, dietary fiber, antioxidants, and mainly polyunsaturated fatty
208 acids. However, in relation to the control formulation, AA levels significantly increased by
209 around 33% with the addition of 5% of chia flour and around 700% with the addition of 10,
210 15, and 20% of chia flour. The higher formation of AA in the samples with chia could be
211 related to the levels of precursors. Chia flour showed a lower content of reducing sugars (1.6
212 g/100 g) but a higher one of free asparagine (42.8 mg/100 g) than wheat flour (respectively
213 of 5.6 g/100 g and 23.4 mg/100 g), leading to an asparagine/reducing sugars ratio of 4.2 for
214 wheat flour versus 26.8 for chia flour. In addition, chia flour presented high levels of
215 dicarbonyl compounds such as methylglyoxal and glyoxal, that were not detected in wheat
216 flour. Dicarbonyl compounds are reactive intermediates of the Maillard reaction, for this, the
217 progressive addition of chia seeds flour has promoted the AA formation during baking.

218 Similarly, the work of Manolache et al. ^[33] evaluated the AA content of wheat flour-
219 based cookies formulated with the addition of 25-100% of wholemeal oat flour. The amount
220 of AA formed during the baking process increased proportionally with the amount of
221 wholemeal oat flour added in the recipe, reaching around 350 µg/kg for oat flour percentages

222 of 75 and 100%. As it was for chia flour, these outcomes could be related to the higher
223 protein, mineral, total fat, sugar, crude, and dietary fiber contents in cookies obtained with
224 wholemeal oat flour than in those with wheat flour.

225 In a more recent study, Sazesh and Goli ^[6] replaced wheat flour with quinoa flour at
226 levels of 25, 50, and 100%, also using different concentrations of sodium bicarbonate,
227 different baking temperatures (160, 185, and 210 °C), and the same time (20 min). The
228 authors applied the response surface methodology with the combined model design expert
229 test (D-optimal design) to obtain cookies with desirable hardness, density, browning index,
230 and low AA content. The two optimized formulas selected, corresponding to the replacement
231 of wheat flour with 72 or 100% of quinoa flour, sodium bicarbonate 0.05%, and baking
232 temperature of 160 °C, showed a drastic decrease of AA when compared to the 100% wheat
233 flour sample. These authors did not determine the sugars content of the used flours, but, based
234 on earlier studies of Maradini Filho et al. ^[34] and Navruz-Varli and Sanlier ^[35], the obtained
235 results were attributed to the fact that quinoa contains about 3% of sugars represented mainly
236 by maltose, D-galactose, and D-ribose, therefore low levels of fructose and glucose which
237 are the most effective reducing sugars involved in the AA formation. In addition, compared
238 to wheat flour, quinoa flour has much lower levels of asparagine, which is the most effective
239 amino acid participating in the Maillard reaction.

240 A large number of flour types for the formulation of cookies were tested by Žilić et
241 al. ^[12]. In detail, refined wheat flour was compared with wholemeal flours of eight genotypes
242 of grain cereals (bread wheat i.e., *Triticum aestivum* var. *lutescens*, durum wheat, soft wheat,
243 hard wheat, triticale, rye, hull-less barley, and hull-less oat) and four genotypes of maize
244 (white-, yellow-, red-colored standard seeded maize, and blue-colored popping maize). The
245 interrelationship between the initial content of proteins, free asparagine in cereal flours, and

246 AA in the cookies, as well as the correlation between contents of AA and free asparagine in
247 baked cookies, were analyzed. Data indicated that hull-less oat, durum wheat, and rye flour
248 contained the highest content of free asparagine (859.8, 603.2, and 530.3 mg/kg,
249 respectively), hence generated the higher amount of AA in cookies baked for 13 min at 180
250 °C. The results confirmed once again that the use of cereal flours low in free asparagine can
251 be an effective strategy for AA mitigation in cookies.

252 On the other hand, contrary to many studies in the literature, Chen et al. [36] found
253 that, despite the higher asparagine content, rice flour when used for cookie formulation
254 involved a lower AA formation, ranging from not detectable to 450 µg/kg, compared to the
255 AA levels found in the cookies obtained with wheat flours, ranging from 155 to 982 µg/kg.
256 In addition, these authors found that cookies made from finely milled rice or wheat flours
257 had substantially lower AA levels than those from respective wholemeal flours grains. The
258 results showed that non-wholemeal flours did not promote an increase in the reducing sugar
259 content of the flours but increased free asparagine, especially for rice flour. Deviating from
260 the mainstream concept, the study concluded that the AA content in cookies was apparently
261 not dependent on the quantities of reducing sugars and free asparagine in the starting flour.
262 To explain the results, it was hypothesized that rice flour can be rich in other amino acids,
263 such as glycine, cysteine, and lysine, that promote competitive reactions. Moreover, a
264 difference in AA could be related to alternative pathway formation involving oils and
265 nitrogen-containing compounds in lipid-rich foods, such as cookies. AA can generate from
266 acrolein, formed mainly through the oxidative degradation of fats, and ammonia (NH₃) that
267 can be already present or formed during the thermolysis of amino acids and proteins. [37]

268 In literature, other authors evaluated the effect of flour extraction on the level of AA
269 in cookies since free amino acids and sugars are not homogeneously distributed into the grain.

270 [16, 38–41] Flour extraction degree represents the total amount of flour obtained from 100 kg of
271 the grain cereal. In general, wholemeal flours products are assumed to have a health-related
272 benefit when compared with products made from white refined flours; however, it is also
273 necessary to consider the influence of flour extraction degree on the formation of toxic
274 compounds in the products in which they are used.

275 Haase et al. [38] compared the effect of wheat flour with 0.55% ash content and
276 wholemeal wheat flour on AA level in cookies. Wholemeal flour resulted in an unchanged
277 AA level but in a significantly higher antioxidant activity when compared with cookies
278 formulated with 0.55% ash content flour. Hence, considering the AA/antioxidant index,
279 wholemeal flour cookies significantly exceeded in quality those obtained with flour with low
280 ash content.

281 The investigation of Mustăţea et al. [39] evaluated four types of wheat flour
282 characterized by ash contents of 0.53% (white flour), 0.44% (white flour), 2.37% (semi-white
283 flour), and 0.88% (dietetic flour). The asparagine content in the tested flours increased with
284 the increase of extraction degree, with the highest asparagine content found in semi-white
285 flour (11.5 g/kg). Accordingly, the AA results showed a good correlation with the ash
286 content, the cookies obtained from flours having the higher extraction degree had the higher
287 amount of AA.

288 In another study, Negoită et al. [16] obtained 20 different cookie formulations by
289 combining three types of wheat flour with different extraction degrees (75-85%, 85-95%, 95-
290 100%) and five types of fat sources. By using the same type of fat, it was noted that the lowest
291 AA values (14.6 to 95.8 µg/kg) were obtained in cookies formulated with semi-white flour
292 with the lowest extraction degree (75-85%). On the contrary, the highest concentrations of
293 AA were obtained using flour with a higher extraction degree, black flour (153.3 to 608.9

294 $\mu\text{g}/\text{kg}$) and dietetic flour (166.0 to 667.7 $\mu\text{g}/\text{kg}$), this is because a greater amount of
295 asparagine is present in the outer layers of grains.

296 In another following research, Negoită et al. ^[40] prepared cookies by varying three
297 types of wheat flours characterized by different ash contents (0.53, 0.44, and 2.37%). In
298 cookies obtained from the different types of flour, the AA level increased as increasing
299 baking time, when water content progressively decreased. In addition, the AA level increased
300 also with the increase in the ash content of the flours, the highest level of 1580.3 $\mu\text{g}/\text{kg}$ was
301 obtained in samples with wholemeal flour with an ash content of 2.4%, followed by samples
302 with white wheat flours (ash content of 0.53 and 0.44%) that had AA levels of 387.8 $\mu\text{g}/\text{kg}$
303 and 308.4 $\mu\text{g}/\text{kg}$ respectively.

304 A more recent study, with the aim of validating a methodology based on high-
305 resolution mass spectrometry for the detection and quantification of AA, evaluated three
306 types of cookies made from soft wheat flour and one type formulated from wheat bran. The
307 highest AA value was obtained in cookies made with wheat bran flour (2373 $\mu\text{g}/\text{kg}$), which
308 had higher concentrations of asparagine (691 mg/kg) compared to wheat flour type 65 (54.5
309 mg/kg). ^[41]

310 To the best of the author's knowledge, only the studies of Anese et al. ^[42] and
311 Bartkiene et al. ^[43] proposed using flours obtained with pre-treated grains or pre-treated
312 flaxseed and lupine to control AA formation during baking. Anese et al. ^[42] studied the
313 influence of a low-temperature long-time pre-treatment as a strategy to reduce AA
314 concentration in short dough cookies. In this study, the whole-wheat grains were subjected
315 to heating at 100 °C for 8 h and then milled. The low-temperature long-time pre-treatment
316 was responsible for a great decrease, up to 42%, in AA levels in the obtained cookies
317 compared to the control samples made with flour from unheated wheat. As the pre-treatment

318 did not cause any change in sugar and asparagine concentrations, the reduction in AA levels
319 has been attributed to a difference in the thermal effect generated in the cookies obtained by
320 using the unheated and pre-heated flours. In fact, as the heating pre-treatment caused a 2%
321 moisture decrease in the flour, less time at the same temperature was required to bake cookies
322 at a similar final moisture content.

323 The potential application of fermented lupine and flaxseed by pure culture of
324 *Lactobacillus sakei*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and solid-state
325 (SSF) or submerged (SMF) fermentation to produce safe and high nutritional value cookies
326 with reduced AA was demonstrated by Bartkiene et al. ^[43]. The obtained flours led to cookies
327 with lower AA compared to samples obtained from not pre-fermented ingredients; the
328 fermentation process decreased asparagine content on average of 67.6 and 80.6% and
329 reduced saccharides content of 18 and 79.4% in flaxseed and lupine, respectively. The most
330 effective AA reduction of 78 and 85% was reached in cookies obtained with flaxseed (SMF)
331 and lupine (SSF) flours pre-fermented by *P. acidilactici*. Significant effects of lupine or
332 flaxseed addition, fermentation method, type of microorganisms, and interaction of these
333 factors on AA concentration in wheat cookies were found.

334 All these results indicate that the source of the flour and its composition also related
335 to the extraction degree after the milling process, play a primary role in determining the AA
336 content in cookies. However, it is important to consider that the nature and quantity of flours
337 alternative to wheat can alter the processability of the dough and impact some important
338 characteristics of bakery products, such as taste, color, texture, density, related to consumers
339 acceptability. ^[6, 28, 44, 45]

340 3.1.2 Sugars

341 Other than flours, sugars are one of the key ingredients of cookies that influence their main
342 desired sensorial quality.^[46] The type, quantity, granulation of sugar used contributes to
343 texture, flavor, sweetness, and color of sweet bakery products.^[46, 47] In addition, besides
344 asparagine, the type and quantity of sugars chosen in the formulation of cookies may also
345 play an important role in AA development.^[44] For this reason, their presence in the cookie
346 dough as ingredients alone or as a component of other ingredients must be carefully
347 evaluated. For an overview, the studies that investigated the effect of sugars on AA levels in
348 cookie products have been summarized in Table 2.

349 Many studies have suggested that the replacement of reducing sugars with sucrose
350 (non-reducing sugar) is an effective way to significantly reduce the AA content in cookie
351 products. Amrein et al.^[48] firstly studied this issue in cookies evaluating the reduction of AA
352 content in gingerbread by replacing the ingredients rich in reducing sugars, such as honey,
353 inverted sugar syrup, and caramel coloring, with sucrose in an amount corresponding to the
354 sum of glucose and fructose present in the previous ingredients. Results showed a 95%
355 decrease in AA content in these cookie samples compared to the control ones due to the
356 reduction of reactive carbonyls for the Maillard reaction.

357 Similarly, Graf et al.^[49] reported an AA content reduction of 70% in industrially
358 produced cookies formulated with sucrose solution instead of inverted sugar syrup (46 vs
359 170 $\mu\text{g}/\text{kg}$ AA).

360 The research of Summa et al.^[50] investigated the kinetics of AA formation and sugars
361 decrease in cookies formulated with sucrose or fructose during baking at 180 °C up to 20
362 min. The use of fructose has led to a greater formation of AA in cookies due to its reaction
363 with asparagine in the first baking period (up to 10 min) following a linear rate kinetic. In
364 contrast, sucrose promoted an exponential kinetic reaction showing that a prolonged heating

365 time is required to break the bond between the glucose and fructose monosaccharides before
366 reacting with the amino acid. The authors also studied the impact of the amount of sucrose
367 added in the cookie recipe. Contrary to expectations, a low AA concentration was found in
368 the samples to which sucrose was added in the highest amount (28%) reaching a similar result
369 of the samples in which sugars were not added. The authors explained that these higher levels
370 of AA could be due to the higher relative concentration of protein and, in particular, the
371 amino acid asparagine, which is considered the limiting factor for AA formation in bakery
372 products. Indeed, an asparagine concentration resulted in higher amounts of AA in the final
373 products. On the contrary, increasing the content of sugar in the formulation is equivalent to
374 a dilution of the flour and thus of the concentration of asparagine. However, this was not the
375 case for the fructose formulation, probably due to its high reactivity in the Maillard reaction.

376 Also, Gökmen et al. ^[51] prepared different cookie doughs by varying the
377 concentration of sucrose and glucose. Because replacing entirely sucrose with glucose
378 adversely affected the cookie structure, a fixed amount of sucrose (7% of the dough) was
379 necessarily included in the recipe. The progressive replacement of sucrose with glucose
380 turned into a drastic enhancement in the AA level up to 50% or more. Under the applied
381 baking conditions (205 °C for 11 min), it was considered that the hydrolysis of sucrose can
382 be very limited. Similar results were observed also by Ramadan ^[46] and Nguyen et al. ^[5].

383 Another study evaluated the effects of cookie formulation in terms of the presence of
384 different sugars (glucose or sucrose) and leavening agents on some risk/benefit indexes based
385 on the concomitant formation of AA and compounds with antioxidant activity. For the same
386 leavening agent, cookie recipes with sucrose showed a higher risk/benefit index compared to
387 samples with glucose indicating that the formation of antioxidant activity compounds does
388 not compensate for that of AA. Glucose compared to sucrose have a higher reactivity in both

389 Maillard and caramelization reactions which enhance both the formation of AA and
390 antioxidant activity. This study, therefore, highlighted the importance of also considering the
391 effect of individual sugar types on the formation of beneficial compounds. [52]

392 The use of blackstrap molasses as an alternative to glucose and sucrose was also
393 evaluated in cookies formulated with different leavening agents [46]. Compared to glucose
394 and sucrose, samples formulated with blackstrap molasses showed a higher AA content
395 ranging from 511 to 740 $\mu\text{g}/\text{kg}$ and from 1260 to 2390 $\mu\text{g}/\text{kg}$ in cookies formulated with 3
396 and 2 g of leavening agent, respectively. Besides the high reducing sugars level, these results
397 were attributed to the low pH value in the dough of cookies prepared with glucose or black
398 molasses compared to other samples prepared with sucrose. [46]

399 Indeed, the findings of Sung and Chen [53], analyzing a very simple cookie dough
400 model, made from wheat flour, sugar, and water, showed that fructose reacted significantly
401 faster with amino acids of flour during the first 10 min of baking inducing AA formation
402 compared to glucose and sucrose. Nevertheless, the authors did not find differences in AA
403 levels after 20 min of baking. This indicates that the sucrose had already been converted to
404 glucose and fructose due to the thermal process before this time.

405 The high reactivity of fructose for AA formation was also confirmed by Miśkiewicz
406 et al. [54], who evaluated the effect of different reducing sugars in low humidity carbohydrate-
407 asparagine model systems comparable to a cookie product. The replacement of fructose with
408 glucose or sucrose caused a decrease in the resulting AA content by 29.8 and 44.1%,
409 respectively. These results were attributed to the low melting point temperature of the
410 different sugars that have an impact on the degree of asparagine-to-AA conversion. In detail,
411 sucrose, due to its high melting point temperature, equal to 184 °C, is the least reactive among
412 the analyzed carbohydrates leading to a lower formation of AA. On the other hand, fructose,

413 having the lowest melting point temperature (between 119 and 122 °C), is the most reactive
414 leading to the highest formation of AA.

415 The recent research of Aarabi and Ardebili ^[55] investigated different combinations of
416 inverted sugar syrup and sucrose to study the formation of AA in rotary mould cookies
417 produced on an industrial scale in a three-zones oven, for the following baking temperatures
418 for zone-1, zone-2, and zone-3, respectively, and baking times: 250-320-350 °C for 7 min
419 and 45 s (condition I); 240, 350 and 380 °C for 7 min and 20 s (condition II); and 230, 380
420 and 410 °C for 7 min (condition III). In detail, three recipes with different combinations of
421 sucrose and invert sugar syrup have been studied during three different time-temperature
422 industrial baking conditions. The results confirmed that either type or level of sugars has a
423 strong influence on AA formation, so that decreasing inverted syrup from 9 to 5% and
424 simultaneously increasing sucrose from 13 to 15%, promoted a reduction of AA formation
425 during the baking process carried out at the tested temperature-time conditions.

426 Another sugar widely used in the special cookies and bakery products formulations
427 is brown sugar. Some bakeries prefer using this ingredient because it is considered to be
428 healthier and gives a unique appearance and flavor. The study by Passos et al. ^[56] concluded
429 that using sucrose and brown sugar allowed to obtain cookies with AA values (139-188
430 µg/kg) lower than the ones obtained using only fructose (256-388 µg/kg).

431 However, brown sugar may contain traces of AA itself due to its production process,
432 for this reason, Shyu et al. ^[57] studied AA formation in cookies prepared with dark brown
433 sugars with high and low AA contents (908 and 140 µg/kg, respectively) instead of sucrose.
434 As could be expected, the higher the initial AA content in dark brown sugar, the higher the
435 amount of AA in the final baked product. The addition of dark brown sugar, as a replacer of
436 sucrose, significantly increased the AA levels, both because the content of reducing sugar is

437 higher in brown sugar than in sucrose, and because brown sugar already contains a certain
438 amount of AA.

439 Other authors have evaluated, as a strategy for AA reduction, the use of alternative
440 high-intensity sweeteners and polyols, increasingly used in place of sugar in bakery products
441 to maintain the glycaemic index low. The research of Garcia-Serna et al. ^[58] aimed at
442 evaluating new cookie formulations with sucrose, maltitol, and stevia as sweeteners to obtain
443 high-quality diet products, also determining the AA contents. The use of maltitol and stevia
444 alone allowed to obtain a significant AA content mitigation of 26.4 and 25% respectively
445 compared to the cookie sample with sucrose. However, under the formulation and baking
446 conditions applied, AA levels were very low even for samples with sucrose.

447 The research of Singh and Kumar ^[59] focused on optimizing the formulation of
448 gluten-free cookies using sugar and fat substitutes, such as acesulfame-k and maltodextrin,
449 thus adding a new and healthy choice to the range of commercially available bakery products
450 for celiac, obese, and diabetic people. The replacement of sucrose and fat content with binary
451 (fat and sugar) substitutes also promoted a strong reduction of AA in the final product (from
452 500 to 320 µg/kg).

453 In addition, Suman et al. ^[60] aimed at investigating how AA concentration may be
454 influenced by bakery-making parameters, including dextrose percentage, within a parallel
455 strategy of mycotoxin mitigation related to wholegrain and cocoa cookie production. The
456 increase of dextrose content contributed to the overall AA increase. When a high dextrose
457 level and a high thermal input were employed (200 °C for 8 min) an AA increase up to 120%
458 was observed (data not shown). On the other hand, a combination of lower dextrose content
459 and moderate thermal input (180 °C for 8 min) may lead to an AA reduction up to 77% (data
460 not shown).

461 Therefore, from the study of the literature, it can be concluded that a reduction in
462 sugar content and a careful choice of sugar types could reduce AA levels in cookies.
463 However, changing the type and amount of sugar is a challenge for the bakery industry
464 because of the many functions that this ingredient has in the process and its effect on the
465 main sensory properties of the baked product especially in terms of color and texture. [46, 48,
466 49, 56-59]

467 *3.1.3 Leavening agents*

468 Leavening, raising, or baking agents, are key ingredients used in sweet bakery dough that
469 cause a foaming action which lightens and softens the finished baked product. [19] Small sweet
470 products such as cookies that bake quickly need a fast-acting leavener that releases the gas
471 before the structure sets. Therefore, to provide the desired uniform pore structure and
472 improved eating quality, chemical leavening agents are normally used in the production of
473 cookies. Furthermore, since cookies are characterized by a high amount of sugars, biological
474 yeasts are usually not recommended as the sugars would inhibit their activity and
475 development. [21]

476 The two major chemical leavening agents used in the manufacture of cookies are
477 sodium bicarbonate (NaHCO_3), called also baking soda, and ammonium bicarbonate
478 (NH_4HCO_3), both systems being decomposed into carbon dioxide gas when exposed to heat
479 during baking. [44] Although NH_4HCO_3 is the most widely used leavening agent, it leads to
480 an indirect increase in the formation of AA in cookies probably because it provides more
481 reactive carbonyls originating from the reaction of ammonia with glucose and fructose
482 present in the dough. Glyoxal, methylglyoxal, and many other formed R-dicarbonyls have
483 been shown to react more rapidly with amino acids than glucose or fructose. [48] For this

484 reason, several studies have been conducted in the literature on its replacement by other
485 leavening agents, as summarized in Table 3.

486 Amrein et al. ^[48] investigated the influence of NH_4HCO_3 added in gingerbread dough
487 in different amounts. The results showed that AA formation in cookies was proportional to
488 its content; when the leavening agent was not used almost no AA was formed, on the other
489 hand, gingerbread, prepared according to the traditional recipe with a leavening agent
490 concentration of 0.8%, contained 501 $\mu\text{g}/\text{kg}$ of AA. When 0.4% of NH_4HCO_3 was added, the
491 AA content decreased by 60% (170 $\mu\text{g}/\text{kg}$), whereas 1.6% led to a strong increase in AA
492 content (880 $\mu\text{g}/\text{kg}$). The same authors also evaluated the influence of NaHCO_3 added in two
493 concentrations (0.83 and 1.67%) as an alternative baking agent to NH_4HCO_3 . Its application
494 reduced the AA content to one-third compared to NH_4HCO_3 both for the concentration of
495 0.83 and 1.67%. However, the pH values of the doughs (from 8.2 to 8.8) were significantly
496 higher in the samples with NaHCO_3 , when compared to those obtained with 0.8% NH_4HCO_3
497 (pH 6.9). These results showed that NaHCO_3 allows the preparation of cookies with a
498 substantially lower AA concentration and that a more alkaline pH does not necessarily imply
499 a higher AA content in gingerbread.

500 Following these previous findings, Graf et al. ^[49] and Sadd et al. ^[61] tested various
501 combinations of baking agents such as NH_4HCO_3 , NaHCO_3 , and tartaric acid ($\text{C}_4\text{H}_6\text{O}_6$), an
502 organic acid often added to baking powders to enhance leavening in sweet bakery products.
503 The amount of each individual compound in each combination was chosen to obtain the same
504 volume of gas released from the standard baking agent composed of 127 g of NH_4HCO_3 , 273
505 g of NaHCO_3 , and 195 g of $\text{C}_4\text{H}_6\text{O}_6$ per 100 kg of dough. The complete replacement of
506 NH_4HCO_3 by NaHCO_3 promoted a reduction of over 70% of AA content. The authors stated
507 that part of this effect on the AA content might also be ascribed to a lower pH related to the

508 presence of more tartaric acid when NH_4HCO_3 was fully replaced by NaHCO_3 .^[49] The pH-
509 dependence of the Maillard reaction exhibits a maximum of AA formation at pH values
510 around 8, on the other hand, lower pH induces a reduction of AA formation^[60, 62]; the effect
511 of the presence of organic acid is discussed below in the section “3.2.1 Organic acids”.

512 Various following studies confirmed that generally, any leavening agent increased
513 AA and that ammonium-based agents gave the highest levels while the replacement with
514 NaHCO_3 as the only baking agent could be a strategy to decrease the AA in shortbread.
515 Moreover, the presence of tartaric acid allows to reduce AA formation inducing the dough
516 pH reduction.^[38, 46, 53, 61, 63, 64]

517 Contrary to previous findings, Courel et al.^[65] observed that AA in cookies appeared
518 to be not affected by the presence or absence of NH_4HCO_3 (0 or 0.33% of dough). According
519 to the authors, this observation may be partly due to the limited number of AA analyses in
520 this study, leading to an insufficient data set for discrimination purposes.

521 A further study tested the effects of recipe composition in terms of leavening agents
522 and sugars contents on a risk/benefit index considering the formation of AA and antioxidant
523 compounds in cookies.^[52] Cookies prepared with sucrose and NaHCO_3 showed a
524 significantly higher index for AA/antioxidants than those prepared with NH_4HCO_3 . These
525 results highlighted that NH_4HCO_3 was not efficient for enhancing the formation of
526 substances with antioxidant activity. The lack of antioxidant formation was not observed in
527 the recipe with glucose and NH_4HCO_3 ; in fact, the presence of glucose probably increased
528 the formation of compounds with higher antioxidant activity compared with sucrose recipes
529 as discussed in the section “3.1.2. Sugars”.^[52]

530 Kukurová et al.^[64] also observed that using sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$) as a
531 leavening agent in cookie formula allowed to obtain a final AA concentration similar to the

532 control sample obtained without leavening agents. However, the author did not provide a
533 possible explanation for this result. Also, in this study, the highest AA levels were found in
534 cookies obtained with NH_4HCO_3 .

535 In the study of Suman et al. ^[60] a predictive model was developed, suggesting a
536 significant role of low pH values of cookie dough, related to the presence of NaHCO_3 as a
537 leavening agent, on the reduction of AA formation in the final product. However, the
538 obtained AA levels were not reported.

539 A more recent study of Sazesh and Goli ^[6] aimed to optimize cookie formulation
540 considering three levels of NaHCO_3 (0.05, 0.10, and 0.15% based on the final dough weight),
541 five wheat, and quinoa flour blends, and different baking conditions. The authors concluded
542 that the amount of AA in cookies was mainly affected by the amount of the leavening agent
543 when wheat flour was used. In particular, at the baking temperature of 185 °C, the AA amount
544 increased with increasing NaHCO_3 and wheat flour in cookie formulation, while the lowest
545 amount of AA was observed with increasing levels of quinoa flour at all levels of NaHCO_3
546 highlighting that the formation of AA is more likely to be influenced by flour rather than the
547 leavening agent. Since at least 0.05% NaHCO_3 raised the pH level to more than nine and
548 quinoa flour compared to wheat flour has a low concentration of asparagine and reducing
549 sugars as AA-producing agents, the AA-producing Maillard reaction occurred to a lesser
550 extent.

551 The choice of type and amount of leavening agents can be a strategy to reduce AA
552 levels in cookies, however, it must be considered that different leavening powders in the
553 formulation can significantly influence the final quality of cookies mainly in terms of
554 textural, physical, and organoleptic characteristics. ^[19] For example, according to Graf et al.
555 ^[49], cookies prepared with a leavening agent without NH_4HCO_3 showed a lesser leavening

556 as compared to the standard product formulated with a traditional baking agent composed of
557 a mixture of NH_4HCO_3 and NaHCO_3 . However, in this study, the difference in leavening
558 capacity was not problematic as the cookies were used as a semi-finished ingredient for other
559 bakery products, and its suitability for further use was not negatively affected. Sensorial
560 analysis carried out on cookie samples indicated that the addition of NaHCO_3 at 1% did not
561 affect the main sensorial proprieties compared to the other formulas prepared with
562 NH_4HCO_3 .^[46] However, Kukurová et al.^[64] and Canali et al.^[19] reported that in general the
563 addition of high doses of NaHCO_3 provides an alkaline taste, a yellowish crumb, and surface
564 coloration and an unpleasant taste, known as “soda bite”. On the other hand, the use of
565 NaHCO_3 must be handled with care, as it increases the sodium content of the formulation,
566 with organoleptic changes and nutritional consequences. Further results revealed that
567 NH_4HCO_3 and NaHCO_3 leavening agents led to a shape expansion and a crispy texture of
568 the cookies when desired, whereas cookies with $\text{Na}_4\text{P}_2\text{O}_7$ were paler, smaller, and harder.^[64]

569 *3.1.4 Oils and fats*

570 Fats and oils are added to the formulations of many bakery products to improve sensory and
571 rheological characteristics; moreover, the presence of fat influences the dough processability
572 and the shelf-life of products.^[66] However, as reported by several studies summarized in
573 Table 4, the type and amount of fats used in the cookie’s formulation can also influence the
574 AA content of the final product.

575 Cookie doughs enriched with three types of virgin olive oils, classified according to
576 their content in phenolic compounds in high, intermediate, and low oleic oils were evaluated
577 in terms of AA formation during baking at 190 °C up to 16 min by Arribas-Lorenzo et al.
578^[67]. No significant differences in AA levels were found among the different cookies for the

579 shortest baking times of 8, 10, 12, and 14 min. However, after baking for 16 min, the use of
580 low oleic oil resulted in the highest level of AA (805 µg/kg) while high oleic oil resulted in
581 an AA value reduced by 20% (637 µg/kg). The same authors also evaluated the impact of the
582 oxidation degree of the oil, using in cookie formulation sunflower oil previously heated at
583 180 °C for 17 h in a laboratory oven compared to the control one. Both samples of cookies
584 showed a significantly different AA content when baked for 8 min and a significantly similar
585 one when baked for 14 min. After 16 min of baking, AA rapidly increased in cookies
586 formulated with oxidized oil reaching levels about 59% higher compared with control one.
587 It can be concluded that the use of oxidized oil in the cookie formulation led to a huge
588 increase in AA formation during cooking, thus the presence of antioxidant compounds is a
589 possible strategy to control AA formation.

590 Anese et al. ^[68] tested the effect of different amounts of margarine (0, 8, and 15%)
591 and alternative fats such as palm oil and monoglyceride-palm-oil-water gel (hydrogel) added
592 in the cookie recipe. Concerning margarine and palm oil, the highest AA concentration was
593 found in the free-fat cookies, while both fats addition significantly reduced (from 41 to 28%)
594 the formation of AA. These data seem to indicate that during baking the presence of melted
595 margarine (transition phase at 62 °C) could hamper the interaction between the precursors in
596 the aqueous phase, leading to lower amounts of AA. However, even if the two fats had
597 different chemical compositions and physical properties, no significant differences in AA
598 formation were found between margarine and palm oil-containing cookies. On the contrary,
599 the substitution of fat with the hydrogel caused a significant increase of AA content, leading
600 to levels comparable to those obtained for the fat-free formulation. This result indicates that
601 the incorporation of palm oil in the form of the hydrogel may modify the “hampering effect”
602 of fat towards AA formation.

603 Another study of Haase et al. ^[38] assessed the AA formation in relation to the fat
604 content used in the cookie formulation to alter volume during baking. It was concluded that
605 a reduction of shortening content by around 40% improved the final volume of cookies.
606 Nevertheless, AA content dropped down non-significantly. However, in this study, the
607 authors did not specify any possible assumptions related to the obtained results.

608 A further in-depth study of Negoită et al. ^[16] focused on the influence of five types of
609 fat, such as sunflower oil, palm oil, margarine, lard, and butter, on the AA content of cookies
610 also formulated with different flours. Although the processing conditions were the same, for
611 each type of flour, the use of the same amount of fat in the formulation with different lipid
612 content, ranged from 60 to 100%, led to an increase in the level of AA with the following
613 trend: margarine < butter < lard < sunflower oil < palm oil. Fats with a high lipid content
614 (100%) like lard, sunflower, and palm oils, provided a higher level of AA compared to the
615 types of fat with less lipid content of 60 and 65%, respectively.

616 For cookies formulated with black wheat flour (85-95% ash content) the use of
617 different amounts of fat with the same lipid content (60%) was evaluated. ^[16] In general, using
618 a smaller amount of fat resulted in a decrease in AA content of 11-15% compared to cookies
619 where the same amount of fat with a different lipid content (60-100%) was used. Thus, the
620 AA content was higher when the fat contained a higher level of triglycerides, mainly with
621 unsaturated fatty acids. Triglycerides are responsible for the formation of AA, probably
622 because they lead to increased formation of acrolein through oxidation. It is known that the
623 formation of AA from the reaction of acrolein and ammonia (NH₃) is a possible secondary
624 route to be considered in fat-rich products. ^[69-72]

625 Sung and Chen ^[53] evaluated the effect on AA formation of adding or not shortening,
626 a very common fat in the formulation of bakery products, in model cookies consisting of

627 flour and water. From 10 to 20 min of baking model cookies with shortening in formulation
628 had a lower level of AA compared to those without shortening. The mitigating effect on AA
629 formation given by the addition of shortening in formulation could be due to their partial
630 hydrogenation that prevents the reaction between the amino acids and the reducing sugars.

631 The previously mentioned authors who studied the influence of oils and fats in the
632 cookie formulation on the formation of AA did not consider, at the same time, their influence
633 on the final quality characteristics of the product, particularly on the overall sensory
634 properties. Thereby, further studies are needed to simultaneously evaluate the influence of
635 fat/oil on both desired final cookie characteristics and AA formation.

636 *3.1.5 Salts*

637 Salts have traditionally been used during the manufacture of bakery products as they cause
638 several important changes in rheological, technological, and sensory parameters.^[44] Sodium
639 chloride (NaCl) is the main salt added as a flavor enhancer and is also used in low quantities
640 in sweet bakery products, including cookies. Monovalent and bivalent ions such as NaCl can
641 influence the development of Maillard reaction through the dehydration of various key
642 intermediate compounds.^[73] Due to the common use of salt in cookie formulation, many
643 researchers, reported in Table 5, have studied its effect on the AA formation in the product.

644 Based on previous studies showing that the addition of polyvalent cations such as
645 Ca^{2+} prevents the formation of AA in bakery products^[74-76], Fiore et al.^[73] evaluated the
646 incorporation of microencapsulated NaCl into cookie recipes in the increasing percentages
647 of 0, 0.32, 0.65 and 1%. It was found that the formation of AA was not significantly modified
648 by the presence of salt. Cookies with 0.65% of NaCl showed an average AA concentration
649 of 278 $\mu\text{g}/\text{kg}$, whereas the control without NaCl had the highest concentration equal to 313

650 $\mu\text{g}/\text{kg}$. These data showed that there was not a direct relationship between NaCl concentration
651 and AA levels in cookies.

652 Van Der Fels-Klerx et al. ^[77] prepared cookies formulated without and with 0.65%
653 NaCl, baked at different temperatures of 180, 190, and 200 °C for 15 min. The results
654 revealed a significant reduction in AA of approximately 16 and 30% in samples prepared
655 with the presence of salt, when baked at the lower temperatures of 180 and 190 °C,
656 respectively. This was attributed to the inhibition of the formation of Schiff's base, which is
657 formed in the condensation reaction between asparagine and reducing sugars, by the release
658 of the two monovalent ions (Na^+ and Cl^-) during baking at 180 and 190 °C. In addition, AA
659 concentration reached the maximum when cookies from both recipes were baked at 200 °C,
660 with no significant differences between them, demonstrating that the effect of salt
661 concentration does not occur at high baking temperatures.

662 In agreement with these results, Sung and Chen ^[53] found a positive action of salt in
663 reducing AA levels when added to 1% in model cookies made from flour and water and
664 baked at 205 °C for 15 min. Specifically, at this baking time, an AA content of 103.3 $\mu\text{g}/\text{kg}$
665 was found in cookies with NaCl and 790.1 $\mu\text{g}/\text{kg}$ in cookies without salt. However, after 20
666 min of baking at the same temperature, no significant differences were found between
667 cookies made with ($739.8 \pm 118.0 \mu\text{g}/\text{kg}$) and without ($953.4 \pm 26.8 \mu\text{g}/\text{kg}$) NaCl.

668 The use of NaCl in the formulation as a strategy to reduce AA in cookies requires
669 further investigation; furthermore, its impact on the organoleptic and health characteristics
670 of the products must also be taken into account, so the choice of the amount used must be
671 undertaken with some degree of care. ^[45] Some authors in the literature have tried to assess
672 the replacement of NaCl with other salts as another useful strategy to reduce the presence of
673 AA in cookies, without increasing sodium intake beyond the amounts recommended by the

674 World Health Organization (WHO). It has been suggested that adding divalent metal ions
675 could promote the stability of the interaction between asparagine and the food matrix at high
676 temperatures (stable polymer network); thereby rendering this amino acid unavailable for
677 reaction with carbonyl precursors to produce AA. [78] To test this effect, Sadd et al. [61]
678 incorporated calcium in cookie dough as chloride (CaCl_2), carbonate (CaCO_3), or propionate
679 ($\text{C}_6\text{H}_{10}\text{CaO}_4$) in the concentration of 2, 1, and 0.7 or 0.35%, respectively. When incorporated
680 into cookie dough, calcium in the form of chloride and carbonate reduced AA by 60 and
681 15%, respectively. On the contrary, calcium propionate, already added to bakery products in
682 the UK as a preservative (up to 0.2%), incremented AA levels of about 15 and 20%,
683 respectively. The reasons for this behavior were not clear for authors because the addition of
684 propionic acid alone had little effect on AA levels, it allowed only a slight reduction of about
685 2% compared to the control cookie. Calcium supplementation seems promising for AA
686 control, but interactions with other ingredients (especially propionate) need further
687 investigation.

688 The study conducted by Quarta and Anese [79] found no changes in AA formation in
689 cookies formulated with 0.25% of CaCl_2 or MgCl_2 compared to the control sample without
690 salt. However, a 60% reduction was achieved when these salts were added in combination
691 (1:1, w/w), suggesting a synergic effect of the cations Ca^{2+} and Mg^{2+} on the AA reduction in
692 cookies. On the other hand, the results showed that the potassium acetate (CH_3COOK) was
693 responsible for the greatest increase in AA of 116% in the cookies compared to the sample
694 with no salt.

695 The effect of calcium derivatives on AA levels in cookies was also evaluated by Açar
696 et al. [80] adding in cookie recipe 0.04, 0.2, and 0.4% of CaCl_2 and calcium salts of lactic acid
697 such as Puracal Act 100 (PA100) and Puracal Act 200 (PA200), characterized by a ratio of

698 calcium to lactate of 23 to 35% and 20 to 44%, respectively. Compared to the control sample
699 without salt, each calcium derivative contributed to a decrease in AA formation in cookies
700 directly related to the amount of calcium added. At the amounts of 0.2%, PA100 and CaCl₂
701 were found more effective to mitigate AA formation in cookies compared to PA200, leading
702 to a reduction in AA of 72.4 and 66.3%, respectively. These results were explained by the
703 presence of less calcium and more lactate in PA200 than PA100, the organic acid may have
704 facilitated the formation of AA by promoting the hydrolysis of sucrose. Moreover, in this
705 study, experimental cookies with and without calcium addition were prepared to determine
706 the effect of calcium on the rate of AA formation during baking. The reaction rates based on
707 the slopes of AA formation indicated that the presence of 0.4% of CaCl₂ significantly reduced
708 the AA formation in cookies. The AA inhibition ratios were found to be 63.7, 74.1, and
709 73.7% at 150, 200, and 250 °C of baking, respectively.

710 Chang et al. ^[81] compared the effects on AA of adding different quantities of various
711 calcium salts such as calcium lactate (C₆H₁₀CaO₆), calcium citrate (Ca₃(C₆H₅O₇)₂), calcium
712 acetate (C₄H₆CaO₄), and calcium carbonate (CaCO₃) plus NaCl in cookies and alone in
713 model cookies prepared with only flour, water, and sucrose. All calcium salts addition has
714 been shown to reduce AA in all samples. The AA concentration of the model and control
715 cookies mainly decreased when fortified with CaCO₃; the addition of a concentration of
716 0.06% (% w/w of dough) of this salt resulted in a reduction in AA of 30 and 13% compared
717 for model and control cookies, respectively. On the other hand, the AA content of calcium
718 lactate-added cookies was significantly lower than that of the corresponding control cookies.
719 The reducing sugar content in the model cookies with calcium lactate was higher than that of
720 those fortified with other calcium salts, confirming the enhance of hydrolysis of sucrose to
721 reducing sugar in presence of organic acid as reported previously by Açar et al. ^[80].

722 The effect of replacing NaCl with different salts such as CaCl₂, potassium chloride
723 (KCl), and two different salt replacements consisting of 13.8 g Na/100 g plus 20.0 g K/100
724 g (SR-I) and 14.3 g Na/100 g plus 17.1 g K/100 g (SR-II) in cookies baked at 190 °C for 20
725 min was studied by Mesías et al. ^[82]. The AA levels detected ranged from 153.4 µg/kg to
726 380.8 µg/kg, with the highest values in cookies containing NaCl, KCl, and the salt substitute
727 SR-I. The AA concentration decreased by up to 17% when NaCl was replaced by SR-II,
728 while reductions of 35 and 40% were observed in cookies formulated with a mixture of NaCl
729 and SR-I or SR-II, respectively. This could be explained by a possible synergistic effect
730 between the salts when they are mixed in the same cookie recipe, in agreement with the
731 observations of Quarta and Anese ^[79].

732 Contrary to the previous finding, in the more recent study of Shyu et al. ^[57], the
733 presence of 1% of calcium ions was not associated with either a reduction in AA formation
734 or an increase in the amount of reducing sugars in cookies formulated with dark brown sugars
735 with high and low AA contents (908 and 140 µg/kg, respectively). Gökmen et al. ^[51] reported
736 that the Schiff base formation was mitigated and changed to another pathway, with the
737 dehydration of glucose generating hydroxymethylfurfural and furfural. The reaction
738 proceeded in this way when calcium ions were increased. However, the results of Shyu et al.
739 ^[57] agree with the mechanistic model based on an asparagine-related pathway proposed by
740 Nguyen et al. ^[83]. Both authors claimed that fructose reacted with asparagine to form a Schiff
741 base without any Amadori rearrangement product or sugar fragmentation before
742 decarboxylation to produce AA.

743 In light of these results, the addition of NaCl or other salts is a possible intervention
744 to minimize AA formation in cookies. However, it must be pointed out that types and/or
745 quantities of some usable salts may be responsible for undesired effects, such as failure in

746 the development of desired sensory properties. ^[61, 80, 81] For example, calcium chloride, when
747 used in certain concentrations, hindered the growth of sweet cookies and the products had an
748 unpleasant taste. ^[61] In addition, the calcium derivatives affected the cookie's surface colors
749 by increasing surface lightness (L*) and decreasing the redness (a*) parameter. ^[80] On the
750 other hand, Chang et al. ^[81] showed that the overall acceptability of the fortified cookies is
751 significantly improved by the addition of calcium carbonate, while other calcium salts had a
752 significant negative effect on the appearance and spread ratio of the product.

753 **3.2 Additional ingredients**

754 *3.2.1 Organic acids*

755 Other minor ingredients such as organic acids, commonly added to bakery products to
756 regulate acidity and improve flavor or leavening, have been tested in numerous studies in the
757 literature for the control of AA formation in cookies. In Table 6, the studies that evaluated
758 the effect of this and other feasible additional ingredients used in cookie formulations for the
759 mitigation of AA formation are reported. It is widely established that pH values can influence
760 the formation of AA. Lowering the pH of a food matrix prevents the nucleophilic addition of
761 asparagine with a carbonyl compound and the formation of the corresponding Schiff base, a
762 key intermediate in the Maillard reaction and thus AA formation. ^[72, 84]

763 Amrein et al. ^[48] carried out various experiments checking the ability of citric acid to
764 reduce AA content in gingerbread cookies. The addition of 0.5 and 1.0% of dough weight
765 resulted in drops of pH to 5.6 and 5.0, and in a reduction of the AA concentration by factors
766 of 4 and 40, respectively.

767 These results are in line with those reported by Graf et al. ^[49], who added tartaric acid
768 in different proportions to the dough of semi-finished cookies, leading to a decrease of AA

769 formation at all additional levels. The use of 0.24% tartaric acid by weight of the dough
770 decreased the AA content by one-third, while an even higher acid addition of 0.29% had a
771 slightly greater effect on the reduction of AA content by 44%, which was not significantly
772 lower than in the previous experiment.

773 Also, Gökmen et al. ^[51] evaluated the addition of citric acid in three different
774 percentages in cookies prepared with sucrose and with glucose plus sucrose, corresponding
775 to doughs pH of 7.40, 4.37, and 3.28 in both recipes. Lowering the pH by adding citric acid
776 to the dough with glucose and sucrose, resulted in a 67% of reduction in the AA content of
777 cookies. However, the addition of citric acid to dough formula with sucrose alone increased
778 the AA formation in cookies, probably due to the excessive hydrolysis of sucrose, which
779 increased the concentration of reactive sugars.

780 The work of Mogol and Gökmen ^[85] showed that the addition of formic acid in cookie
781 dough did not significantly affect the formation of AA at all baking temperatures studied.

782 Another more recent study by Passos et al. ^[56], tried to modulate the AA formation in
783 cookies by adding polymeric acid compounds such as galacturonic acid (monomer) other
784 than pectin (polymer) or partially hydrolyzed pectin (oligomer) as substitutes to tartaric acid.
785 When using the monomer galacturonic acid, which consists of both a reducing sugar and an
786 acidic part, the formation of AA and its mitigation were simultaneously achieved.
787 Consequently, when 1% of this monomer was added to the cookie dough, the AA formation
788 increased by 95% compared to the control sample without pectic polysaccharides. When
789 using 1 or 5% of partially hydrolyzed pectin, the amount of AA in cookies was significantly
790 lower than galacturonic acid-based cookies but comparable to the amount of AA observed in
791 the control cookies. The partially hydrolyzed pectin although presenting 13.8% of reducing
792 sugars, which had a net contribution to the formation of AA, had also an AA mitigation effect

793 due to the pH-lowering outcome of the repeating units of galacturonic acid residues in its
794 oligomeric structure. Using 1% of pectin, which contains a lower amount of reducing sugar
795 compared to galacturonic acid, the AA level was significantly lower than that of cookies with
796 galacturonic acid but still comparable to the amount of AA observed in the control and
797 partially hydrolyzed pectin cookies. Nevertheless, although having the same galacturonic
798 acid content, 1% pectin samples contained half of methyl-esterified residues, which did not
799 contribute to the same extent of pH-lowering effect of galacturonic acid and partially
800 hydrolyzed pectin samples. In conclusion, the authors of this experiment suggested that the
801 lowest amount of reducing sugars of cookie samples with pectin is the cause of the lowest
802 formation of AA when compared with the other samples. Furthermore, this hypothesis is also
803 corroborated by the experiment with the addition of pectin by 5%, where the AA formation
804 was significantly reduced by 67% compared to the control without pectin addition. Similarly,
805 the addition of only 1% of tartaric acid, which contributes to the acidifying effect without
806 adding reducing sugars towards AA formation, promoted a 52% decrease in AA content
807 compared with the corresponding control. In addition, a 5% of tartaric acid addition to the
808 dough resulted in an 81% AA content reduction.

809 In summary, the addition of some organic acids is a possible way to control the AA
810 level in cookies. However, the amount of acid must be carefully assessed evaluating its
811 effects on sensorial and physico-chemical properties of the cookies.^[48, 51, 56] The addition of
812 citric acid in gingerbread led to cookies with a clearly acidic taste, not homogeneous color
813 surface, and insufficient volume which limited their acceptability.^[48, 51] Cookies prepared
814 with tartaric acid and examined by an untrained sensory panel showed a difference in
815 hardness, crispness, and firmness to the touch compared to those prepared without any
816 organic acid; a harder, brittle, and sandy texture on the tongue and a sour taste.^[56]

817 *3.2.2 Amino acids*

818 Another strategy to control the formation of AA in bakery products is the use of different
819 amino acids that can compete with asparagine in the Maillard reaction or that can react with
820 the nucleophilic amino group of AA formed through Michael addition reaction, promoting
821 its elimination.^[44, 86] In the literature, different types of amino acids and certain protein
822 isolates have been tested in cookies formulations to control AA (Table 6).

823 The first study of Amrein et al.^[48] did not find a reduction in AA contents in
824 gingerbread cookies with the addition of the amino acids L-glutamine, L-lysine, or glycine
825 at a 0.2% of dough weight. For glycine, a concentration of 1% was also evaluated which
826 reduced the AA content in cookies to one-third. Another tested amino acid, L-cysteine,
827 showed a tendency to reduce the dough pH and AA content at the concentration of 0.05 and
828 0.2%.

829 Salazar et al.^[87] in an attempt to investigate additional beneficial properties of the
830 underexploited plant, investigated the AA mitigating effect of amaranth proteins isolate used
831 in cookies formulation. The addition of amaranth protein isolates significantly decreased the
832 AA formation in the cookies upon baking. In particular, AA mitigation was reduced from
833 89% (using a baking time of 7 min, which was the optimum baking time for the assayed
834 cookies) to 26% (with a baking time of 9 min). This result is due to the amino acid
835 composition of amaranth proteins that are rich in lysine (4.8-6.4 g/100 g of protein) and sulfur
836 amino acids (3.7-5.5 g/100 g of protein).

837 Another study tested the effects of cysteine plus glycine amino acids or in
838 combination also with CaCl₂ on the reduction of AA formation in cookies. All the added
839 compound mixtures reduced the contents of AA, showing a synergistic effect when amino
840 acids were combined with each other or with CaCl₂. The optimal formulations were 0.36%

841 of cysteine plus 0.2% glycine plus 0.06% CaCl₂ and 0.29% cysteine plus 0.2% glycine which
842 led to a drastic AA reduction of 97.8 and 98%, respectively. ^[88]

843 As regards protein-based ingredients commonly added in the formulation of cookies
844 such as milk and egg, Suman et al. ^[60] that tested different recipes obtained from an
845 experimental design, based on the overall statistical evaluation, found no differences in
846 mitigation effect on wholegrain and cocoa cookies baked at pilot-plant processing conditions
847 (data not shown). This result was attributed to the fact that the ranges of variation studied for
848 milk and egg content in the recipe formulation were very close, 5-8 and 4-7% respectively.

849 It has been demonstrated that the addition of certain amino acids, in supplement of
850 asparagine, to the formulation of cookies can reduce the AA content. However, even for these
851 ingredients, it is necessary to evaluate possible modifications on the sensory proprieties of
852 the final products. For example, the ready reaction between glycine and reducing sugars
853 strongly increases the browning of the cookies' surface, as more melanoidins result, while
854 the amino acid L-cysteine has an unpleasant taste and odor presumably caused by S-
855 containing decomposition products. ^[48] Nevertheless, to minimize these adverse effects, the
856 amount of the amino acids added can be reduced and these compounds can be used in
857 combination, or natural protein ingredients, such as amaranth protein, can be employed. ^{[87,}
858 ^{88]} However, a much more detailed sensory evaluation of cookies obtained with these
859 ingredients is needed.

860 *3.2.3 Enzymes*

861 Among the most effective mitigation strategies tested for reducing AA in the most at-risk
862 foods, including cookies, there is the use of the enzyme asparaginase (Table 6). This enzyme
863 can hydrolyze asparagine into aspartic acid; hence it represents a good way to remove this

864 AA precursor from the primary ingredients before thermal processing. The effectiveness of
865 asparaginase depends on its concentration, time, and temperature of its incubation, water
866 activity of the food matrix that affects its mobility, and the pH at which the asparagine
867 conversion reaction takes place.^[45]

868 From its use in the cookies formulation, excellent results were achieved leading to an
869 AA reduction of up to 55% in gingerbread baked at 180 °C for 3 min plus 190 °C for 7 min.
870 ^[48] Analysis of the fresh dough treated with asparaginase revealed that it still contained 22
871 mg/kg of free asparagine and that 75% of the total free asparagine had been degraded, which
872 explains why AA formation was not fully inhibited. Its incomplete hydrolysis was probably
873 due to the limited mobility of both the enzyme and the substrate within the cookie dough.

874 Hendriksen et al.^[89] used asparaginase from the fungus *Aspergillus oryzae* in
875 semisweet and ginger cookies formulations. A clear reduction of AA levels was observed in
876 the semisweet cookies, especially with increasing the amount of the enzyme. Treatment with
877 525 ASNU/kg of flour (one ASNU is defined as the amount of enzyme that produces 1 µmol
878 of ammonia per minute at 37 °C and pH 7.0) and a dough resting time of 15 min resulted in
879 AA reduction of 65% when compared to the control sample, while cookie treated with twice
880 the enzyme amount and the same resting time had a reduction in AA content of 84%. For all
881 asparaginase levels, the AA reduction effect increased when resting time was longer than 30
882 min, illustrating that the system operated within the dynamic response range of both enzyme
883 dosages and resting times.

884 A clear enzyme dose-dependent reduction in AA levels was confirmed also by Huang
885 et al.^[90] evaluating the effect of asparaginase produced from *Rhizomucor miehei* in cookies.
886 Approximately 15 and over 80% of AA reduction was reached when the concentration of
887 this enzyme was 0.5 and 100 U/g flour, respectively.

888 The importance of dough water content on the asparaginase activity and asparagine
889 mobility was further investigated by Hendriksen et al. ^[89] in ginger cookies prepared with
890 different water contents and a constant enzyme dose of 1000 ASNU/kg of flour. AA levels
891 measured in the control cookies without the addition of asparaginase were rather constant,
892 despite the differences in dough water contents. For the other samples, a clear correlation
893 between cookies' AA level and dough moisture content was observed. Increasing water
894 content from 11 to 19% with the same concentration of asparaginase, allowed an increase of
895 AA reduction from 34 to 90%. This result was attributed to a limited enzyme-substrate
896 contact in the low-water doughs because of limited diffusion, confirming the previous
897 hypothesis of Amrein et al. ^[48].

898 A further study of Anese et al. ^[68] studied the influence of the matrix composition and
899 structure on the capacity of asparaginase to reduce AA formation in short dough cookies. In
900 detail, formulations differed for water (10 to 20% on total weight) and fat (0 to 15% on total
901 weight) contents, moreover fat type (margarine, palm oil) and lipid phase distribution were
902 considered. The results showed that high water contents, by favoring reactants mobility,
903 promoted AA formation as well as, the enzyme capability, when added to the formulation,
904 of reducing asparagine levels in the final product. Thus, when present, the asparaginase
905 enzyme was responsible for a 58% AA reduction in the sample with the highest water amount
906 of 20%. On the contrary, the presence of fat significantly reduced both enzyme activity and
907 AA development compared with the fat-free cookie formulation, suggesting that fat would
908 make more difficult the contact between reactants. In fact, the highest AA concentration was
909 found in the fat-free cookies and the percentages of AA reduction caused by the addition of
910 the enzyme to the doughs decreased progressively as the fat concentration increased (69, 62,
911 and 58% AA reductions corresponding to 0, 8 and 15% of fat). In addition, the asparaginase

912 capability to lower AA formation seemed to be influenced also by the different structures of
913 systems due to the presence of a different type of fat such as margarine, palm oil, and
914 hydrogel. The AA reduction in the hydrogel-containing cookies (66%) was significantly
915 higher compared to margarine (58%) and palm oil-containing (58%) formulations. Being
916 water-soluble, asparaginase would be confined in the aqueous domain of the hydrogel
917 together with AA reactants. Therefore, probably due to the higher proximity between the
918 enzyme and substrate in the hydrogel system, asparaginase efficiently mitigated AA
919 formation like in the fat-free system. However, the AA level in the hydrogel containing
920 cookie treated with asparaginase was still higher than those found in the margarine and palm
921 oil systems due to the reasons explained above in section “3.1.4 Oils and fats”.

922 In another study, Anese et al. ^[91] also evaluated the effect of asparaginase in AA
923 reduction in shortbread cookies by preparing, according to a three-factor, three-level cube
924 central composite design, 15 recipes different in asparaginase concentration and incubation
925 temperature and time, from 100 to 900 U/kg of flour, 20 to 54 °C, and 10 to 30 min,
926 respectively. In agreement with the results reported previously, the variable that showed the
927 biggest effect in reducing AA was the concentration of asparaginase, followed by the
928 incubation temperature, while the incubation time of the asparaginase infused dough seemed
929 to be the least effective variable. Within the ranges considered in this study, the intermediate
930 asparaginase concentration of 500 U/kg combined with the lowest temperature and 20 min
931 of incubation resulted in the lowest AA formation of 90 µg/kg in short dough cookies.

932 The results of Haase et al. ^[38] demonstrated once again that AA in cookies was
933 significantly reduced when asparaginase was added to the dough and the thermal input was
934 the most relevant criterion. Samples produced without enzymatic treatment showed an
935 exponential increase in AA with increasing baking temperatures. On the other hand, the

936 temperature-related increase in AA in asparaginase-treated samples was on a linear basis
937 indicating that the benefit of the enzymatic activity was especially pronounced at higher
938 thermal input.

939 As already mentioned, a further aspect to consider in order to achieve maximum
940 efficiency of asparaginase application is the pH of the dough which may be modified also by
941 the type of chemical leavening agent used in the cookie formulation. By changing the
942 asparaginase incubation time and pH of the cookie dough by varying the type of leavening
943 agent, Kukurová et al. [64] observed that a deviation of the pH out of the optimal range (about
944 pH 7) for the action of the enzyme leads to a strong limitation in its activity. The highest AA
945 reduction efficiencies of 66 and 75% in the cookie baked at 205 °C for 11 and 15 min
946 respectively were achieved with a 60 min enzymatic pre-treatment at pH 6.78 which is close
947 to the optimum pH value for asparaginase activity. While shifting of pH to 8.10 diminished
948 the asparaginase efficiency to about 50% of AA reduction after 60 min of incubation. To
949 assess whether the required results can be obtained, it is necessary to test prolonging the
950 enzyme incubation or increasing the enzyme dosage.

951 A clear advantage of using the asparaginase enzyme, compared to the other strategies
952 for the AA formation control in cookies, is the low impact it has on the sensory characteristics
953 of the final product. In evidence, some authors found that taste and color of cookies prepared
954 with asparaginase were almost identical to those of the standard product. [48, 68, 91]

955 *3.2.4 Antioxidants*

956 Other minor ingredients studied for the AA mitigation in cookies, as reported in the literature,
957 are represented by antioxidant compounds. Antioxidant compounds can react with AA
958 precursors or intermediates, which may inhibit the overall rate of the Maillard reaction. In

959 particular, they could control AA formation in three ways: by trapping of carbonyls,
960 reduction of sugar degradation through Maillard reaction processes, and radical scavenging
961 activity.^[92, 93] Some antioxidants ingredients and/or certain plant powder or extracts were
962 able to reduce AA formation in cookies, while others showed no effect or even an enhancing
963 effect (Table 6).

964 Significant AA reductions in cookies have been demonstrated by Zhu et al.^[94] adding
965 raw powders and crude aqueous extracts of many different common dietary plants such as
966 cinnamon, clove, coriander, cumin, turmeric, red onion, and some phenolic compounds
967 including cinnamaldehyde, curcumin, and eugenol. Among all plant-based raw materials
968 tested, clove bud powders at various addition concentrations (0.25-4%) showed the highest
969 AA reduction rates in the range of 21.6-41%. For all powders, the inhibitory effects at higher
970 concentrations (from 2 to 4%) were not as marked as those at lower concentrations (from
971 0.25 to 1%). The aqueous extracts from the selected plant materials had slightly better
972 efficiency in reducing AA, with an average decrease of 30.2% compared to the crude
973 powders tested (26.7%). As well as with raw powders, plant extracts decreased AA content
974 in cookies to varying degrees in a nonlinear dose-dependent manner, and the most effective
975 in controlling AA was clove bud extract with reduction rates from 25.1 to 50.9% at different
976 levels of 0.25-4% addition. Data for the three pure phenolic compounds (cinnamaldehyde,
977 curcumin, and eugenol) showed slightly greater efficacy in reducing AA compared to crude
978 plant powders (26.7%) with an average decrease of 29.8%, but comparable levels to aqueous
979 extracts (30.2%). Eugenol promoted less reduction in AA (31.6%) than aqueous extract of
980 clove buds (40.7%), suggesting that other factors than the major additional phenolic
981 constituents in plant materials might be influential in AA formation. For example, crude
982 aqueous extracts usually had strong hygroscopicity and might alter water activity levels in

983 cookies during heating thus affecting AA content. Crude aqueous extracts also contain other
984 food ingredients such as protein and peptides, non-reducing saccharides, and low levels of
985 monovalent/divalent cations, which might also play a role in the AA formation, as explained
986 in the previous sections of this review.

987 Similarly, Li et al. ^[95], evaluating different antioxidant concentrations from 0 to 0.1%
988 of bamboo leaves added in cookie dough, found that the highest AA inhibitory rate of 63.9%
989 was achieved by the antioxidant concentration of 0.02%. This result indicated that after a
990 first positive effect, a threshold value was reached, and increasing the concentration of
991 bamboo leaves antioxidants beyond this value, a negative effect was found. This is the so-
992 called "antioxidant paradox" since polyphenol-rich bamboo leaves can reduce free radicals
993 and reactive free electrons that cause a rapid conversion of asparagine to AA, but on the other
994 hand, a high concentration of antioxidants did not suppress AA formation as it did with a
995 lower dose. Based on these considerations, the same authors evaluated the potential
996 effectiveness of other antioxidants such as sodium erythorbate, tea polyphenols, vitamin E,
997 and tert-butyl hydroquinone added in 0.01 or 0.02%. Results showed that the addition of
998 these antioxidants significantly mitigated the formation of AA in cookies up to 43.0, 71.2,
999 54.1, and 49.6%, respectively. The difference in the inhibitory effect of these antioxidants
1000 was attributed to their antioxidation and polarity diversity.

1001 Ten other pure and partially pure plant polyphenolic compounds (caffeic acid,
1002 chlorogenic acid, European cranberry bush juice (ECJ), ellagic acid, epicatechin, oleuropein,
1003 olive mill wastewater (OMWWE), pomegranate peel (PPE), punicalagin, and tyrosol) were
1004 tested on AA formation in cookies by Oral et al. ^[96]. All of them slightly decreased the AA
1005 formation in cookies at levels between 10.3 and 19.2% in comparison with the control sample
1006 obtained without antioxidants addition.

1007 Passos et al. ^[97] investigated the impact of four instant coffee fractions differently
1008 obtained, by simple centrifugation (WSn), ethanol precipitation (fraction ethanol-soluble
1009 EtSn and fraction precipitated EtPp), or ultrafiltration (fraction HWSn) as ingredients for
1010 antioxidant-rich cookies. The impact of 0.5, 2.3, and 4.6% w/w relative to the dough
1011 (corresponding to 1, 5, and 10% w/w flour) of coffee melanoidin-rich fractions
1012 supplementation on the cookies was evaluated also in terms of AA formation. The content of
1013 AA in the coffee fractions was negligible and did not account for the final level of AA in the
1014 baked cookies. The highest content of AA in cookies was observed for the addition of 2.3%
1015 of EtSn fraction (274 µg/kg). This result is supported by the highest percentage of
1016 monosaccharides (18.9%) precursors in AA formation. On the other hand, the addition of
1017 2.3% of EtPp resulted in a significant AA decrease (31%) when compared to control. This
1018 effect is supported by the lower percentage of monosaccharides (4.8%) of EtPp fraction. No
1019 differences to control in AA contents were observed for any fraction at 4.6%
1020 supplementation. The differences observed between 2.3 and 4.6% addition may result from
1021 a complex balance between AA formation and mitigation opposite effects. AA mitigation,
1022 on the other hand, maybe explained by the scavenger ability of the antioxidants towards the
1023 radical fragments of hydrocarbons that are formed during baking, preventing the formation
1024 of carbonyl groups by lipid peroxidation.

1025 Miśkiewicz et al. ^[98] evaluated the positive effects of dough supplementation with
1026 different amounts of freeze-dried aqueous rosemary extract on AA content in shortcrust
1027 cookies, baked at 170 °C in dry or humid (90% RH) air. The addition of rosemary extract to
1028 the cookie dough resulted in an AA reduction proportional to its concentration in both baking
1029 methods. Compared with products without the antioxidant extract, the greatest decrease was

1030 observed in cookies containing 0.5% of the extract, with a reduction of 18.4 and 15.8% for
1031 dry and moist air baking conditions, respectively.

1032 Again, evaluating two different baking conditions, in conventional and microwave
1033 ovens, AL-Ansi et al. ^[93] proposed the addition of fine fennel and black cumin seeds as a
1034 promising strategy for AA reduction in cookies. The addition of black cumin seeds in the
1035 formulation gradually decreased the AA content by 17-53% in conventionally baked cookies
1036 and by 23-68% in microwave-baked ones. Meanwhile, the addition of fennel seeds
1037 significantly decreased AA to the minimum limit of the quantitation in microwave-baked
1038 cookies and up to 78% in conventionally baked cookies. These results were attributed to the
1039 high antioxidant activity of the seeds, highlighting a potential plant antioxidant source and
1040 mitigation strategy for AA reduction.

1041 Two recent studies have evaluated the addition of ginger ^[99] and of green and spent
1042 roasted coffee samples ^[100] on AA formation in cookies. Ground freeze-dried ginger added
1043 in different amounts of 1, 3, 5, and 7% (w/w of dough) in cookie formulation was able to
1044 significantly reduce the AA content. The AA inhibition rate was 6.2 at the 1% ground ginger
1045 level, and 15.6, 19.1, and 23.7% at the 3, 5, and 7% ginger levels, indicating a dose-dependent
1046 relation. This result was attributed to the phenol hydroxyl group of gingerol that plays a more
1047 important role in the reaction of AA formation than the side chain. In addition, ginger
1048 contains bioactive constituents that alleviate protein glycation by trapping glucose thermal
1049 decomposition product called methylglyoxal, which might affect the inhibition of AA
1050 formation. However, more studies should be carried out on the mechanism of this ingredient
1051 in AA reduction. ^[99]

1052 Cookies obtained with flour fortified with different percentages (3-12% w/w) of spent
1053 roasted coffee (RGCS) and spent unroasted green coffee (UGCS) evaluated by Desai et al.

1054 ^[100] recorded low AA in the range of 32.6-37.8 µg/kg and 23.4-29.7 µg/kg, respectively. This
1055 low AA content was associated with the phenolic antioxidant compounds present in spent
1056 coffee. However, the authors did not determine the content of AA in control cookies obtained
1057 without the addition of both RGCS and UGCS ingredients.

1058 Troise et al. ^[101] demonstrated that cookies enriched with polyphenol powders from
1059 virgin-olive oil mill wastewater (OMWP), rich in secoiridoids, showed a lower concentration
1060 of AA in comparison to control cookies. Specifically, the addition of 0.05 and 0.1% OMWP
1061 resulted in a reduction of AA to 47 and 55%, respectively. However, for 0.2% OMWP
1062 samples there were no significant differences in AA concentration when compared to the
1063 control cookies. ^[101] The authors did not give a specific explanation for the effect of OMWP
1064 concentration, but based on other studies, given their chemical nature, the use of secoiridoids
1065 for the reduction of AA and other highly reactive amides is controversial. ^[102-104]

1066 The inhibitory effects of glutathione (GSH), a tripeptide with antioxidant properties,
1067 consisting of cysteine and glycine, on AA in cookie model systems were investigated by Zhu
1068 et al. ^[105]. The presence of GSH in cookies inhibited the AA formation but without a
1069 proportional relationship between the GSH level and the AA inhibition ratio. No significant
1070 inhibition on AA formation was observed when the GSH amounts were 0.0002-0.001% of
1071 weight dough. However, for other tested GSH levels ranged from 0.002 to 0.01%, the AA
1072 decreasing ratios were in the range of 21-48% compared to the control samples (no GSH
1073 added). The addition of 0.002% GSH showed the best inhibitory effect and decreased the AA
1074 concentration by 48%. Additionally, monitoring GSH and asparagine concentrations it turned
1075 out that after baking, only 6-17% of the initial amount of GSH remained in the cookies and
1076 meanwhile, the residual asparagine levels in the cookies gradually increased with the increase

1077 of the GSH adding amount. This result indicated that GSH participated in the Maillard
1078 reaction and competitively react with glucose against asparagine.

1079 Many of the antioxidant ingredients studied in the literature are plants or spices, so in
1080 some works, their impact on the sensory characteristics of cookies has been evaluated.^{[93, 95,}
1081 ^{99, 101, 105]} For example, a sensorial panel evaluation results showed that color, texture, and
1082 flavor of cookies processed with either bamboo leaves (0.2 g/kg) or vitamin E (0.1 g/kg) did
1083 not differ significantly from control cookies. Nevertheless, other ingredients such as
1084 polyphenols from OMWP are characterized by bitterness and astringency, especially when
1085 added at high concentrations.^[101] Therefore, in parallel with the reduction of AA, it is
1086 necessary to make a careful organoleptic evaluation of final products according to the type
1087 and quantity of ingredients added and kind of cookie.

1088 *3.2.5 Other ingredients*

1089 The industry of bakery products is constantly evolving to offer healthier and environmentally
1090 friendly alternative products that provide consumers an improved nutritive quality.
1091 Therefore, some by-products and fermented ingredients were used to enrich and diversify the
1092 cookies formulations, and the formation of toxic compounds was also evaluated (Table 6).

1093 For example, coffee silverskin can be used in the preparation of functional bakery
1094 products. This by-product of roasting coffee is natural coloring and rich in dietary fiber,
1095 which makes it a good candidate for improving the overall quality of cookies.^[106] Garcia-
1096 Serna et al.^[58] aimed to evaluate the usefulness of Arabica coffee silverskin finding that the
1097 addition of this ingredient did not inhibit AA formation. Moreover, cookies with silver coffee
1098 skin extract, made by boiling in water and drying, had an AA content of 205.9 µg/kg dry
1099 weight which was significantly higher than that found in the control cookies. This is probably

1100 because coffee silverskin extract contained 11.4 $\mu\text{g/L}$ of AA, although this level is
1101 approximately 10 times lower than that reported in coffee beverages (175-263 $\mu\text{g/L}$) by Food
1102 and Drug Administration.

1103 Another coffee by-product is the spent coffee grounds (SCG) obtained after beverage
1104 extraction, including those obtained from instant coffee. Martinez-Saez et al. ^[107] evaluated
1105 the use of SCG from instant coffee as an ingredient in cookie formulation also analyzing AA
1106 levels. Results showed that SCG presented a low concentration of residual AA (37.2 $\mu\text{g/kg}$)
1107 and was a natural source of antioxidant insoluble fiber, essential amino acids, low glycaemic
1108 sugars, resistant to thermal food processing and digestion process, and totally safe. However,
1109 this coffee by-product did not affect AA levels in cookies formulated also with stevia and
1110 oligofructose; AA values were 166 $\mu\text{g/kg}$ and 169 $\mu\text{g/kg}$ when SCG was added or not added,
1111 respectively. Therefore, the results seem to indicate that SCG does not influence the
1112 formation of AA during baking.

1113 Troise et al. ^[108] studied the impact of rapeseed press-cake (RPC), a by-product of
1114 rapeseed oil production, rich in proteins and fiber on the formation of AA in cookies. RPC
1115 was added in different forms to cookie model systems, as cold-pressed RPC, RPC fiber
1116 isolate, and RPC alkaline extract. The addition of cold-pressed RPC led to a significant
1117 increase of AA up to 66.9% in the cookies that was attributed to its high content of AA
1118 precursors, such as glucose and crude protein that could actively contribute to the final
1119 concentration of AA. In addition, considering that also the fatty acid composition affected
1120 AA levels, as cold-pressed RPC is rich in monounsaturated fatty acids and polyunsaturated
1121 fatty acids it is likely that more AA is formed due to the formation of AA via two pathways:
1122 the Strecker degradation of N-(1-deoxy-D-fructos-1-yl)-L-asparagine and the reaction of
1123 asparagine with lipid oxidation products from fatty acids. On the contrary, AA concentration

1124 was reduced to 39.6% in presence of the alkaline extract and down to 4.4% in the presence
1125 of 5.2% of fiber extract. The reduction of AA in cookies containing the alkaline extract can
1126 be ascribed to the direct elimination of AA through Michael's addition of nucleophilic amino
1127 acids, particularly the thiol group of the cysteine side chain. In addition, AA precursors may
1128 react with polyphenols present in the protein extract.

1129 An additional study aimed to investigate the effect of the addition of the vegetable
1130 Jerusalem artichoke (JA) fermented with different lactobacilli (LAB; *Lactobacillus sakei*
1131 KTU05-6, *Pediococcus acidilactici* KTU05-7, and *Pediococcus pentosaceus* KTU05-9) by
1132 solid-state fermentation (SSF) or by submerged fermentation (SMF) on AA content in
1133 cookies. The fermentation technologies were able to reduce AA levels in cookies to different
1134 extents. In particular, the addition of LAB fermented by SMF promotes higher AA reduction
1135 due to lower acidity and higher protease and alpha-amylase activities compared to the
1136 application of SSF. Therefore, fermentation of JA with selected LAB could be the method of
1137 choice to minimize the AA content in cookies without adversely affecting the nutritional
1138 quality, safety, and sensory attributes, including color and flavor, while maintaining
1139 consumer acceptance.^[109]

1140 Another very promising minor ingredient is the hydrocolloid chitosan, a popular
1141 natural food preservative due to its antibacterial and antifungal activities. It may be used in
1142 products subjected to thermal processing as an AA mitigation strategy, due to the availability
1143 of its amino groups to compete with the amino group of asparagine.^[53, 57, 85] Mogol and
1144 Gökmen^[85] investigated the effect of chitosan and formic acid solutions on the formation of
1145 AA in cookies, however, they did not significantly affect the AA formation at all considered
1146 baking temperatures. Nevertheless, it was clear the necessity to also consider the pH-

1147 lowering effect of the acidic solutions in which chitosan is solubilized when determining the
1148 AA mitigation mechanism.

1149 Accordingly, the 1% of chitosan addition was not effective on AA reduction in brown
1150 sugar cookies. ^[57] In contrast to these findings, Sung and Chen ^[53] found significant
1151 mitigation of AA in cookies enriched with chitosan after 15 min baking time.

1152 A methodological approach for the incorporation of other food hydrocolloids such as
1153 gum Arabic (GA), pectin, and carboxymethylcellulose (CMC) in the cookie dough to
1154 investigate the formation of AA in ammonia cookies was applied by Mousa ^[110]. Results
1155 revealed that the use of 0.03% GA in the dough reduced significantly AA content up to 58.6%
1156 compared to the control cookies baked at 180 and 200 °C. The reasons for this behavior could
1157 be due to the gelling or thickening effect of GA on the texture modification of cookies which
1158 consequently could interfere with the molecular interactions between fructose and asparagine
1159 as precursors of AA formation. Moreover, the acidic pH value of GA solution (pH = 4.9)
1160 could be another factor to facilitate the reduction of AA formation in cookies. Contrary to
1161 GA, the use of pectin and CMC at all tested concentration levels did not significantly affect
1162 the AA formation at all temperatures compared to the control cookies.

1163 A recent study investigated the incorporation of passion fruit epicarp flour (PFEF) up
1164 to 9% as a source of high nutritional value into cookies by also assessing the AA content. As
1165 PFEF was added the AA content of cookies considerably increased, attributed to the content
1166 of reducing sugars in PFEF. The highest AA content of 228.4 µg/kg was reached in the
1167 cookies prepared with 9% of PFEF, however, this AA value is lower than the European
1168 standard (350 µg/kg). ^[111]

1169 **4. Conclusion and future directions**

1170 The presence of AA in widely consumed foods including cookies and other bakery products
1171 is currently a challenging issue due to its carcinogenic, mutagenic, and reproductive
1172 toxicological effect on humans. In addition, global regulatory authorities and institutional
1173 communities are becoming increasingly restrictive on the levels of AA allowed in the final
1174 products and its control throughout the food production processes.

1175 Several strategies to control the level of AA in cookies have been extensively
1176 evaluated in the actual literature. Given the wide variety of traditional, innovative, and
1177 usually complex formulations of cookies, it is necessary to evaluate the effect of each type
1178 of major or minor ingredient on the formation of AA during baking. Recipe optimization is
1179 a crucial factor for the control of AA levels in cookies, as the reduction of its formation can
1180 be achieved mainly by:

- 1181 • selecting ingredients with low asparagine and reducing sugar content, such as refined
1182 cereal flours, pseudo-cereal flours (e.g., quinoa), pre-fermented cereal flour, white
1183 sucrose, and alternative sweeteners (e.g., stevia);
- 1184 • adopting the lowest amount of leavening agent, preferring NaHCO_3 instead of
1185 NH_4HCO_3 and combination of leavening agents for example NaHCO_3 plus
1186 NH_4HCO_3 or NaHCO_3 plus tartaric acid;
- 1187 • adding an adequate amount of fat, choosing oils with a high polyphenol content, low
1188 oxidating degree and not exposed to heat, using fats with low lipid content such as
1189 margarine and butter;
- 1190 • using monovalent or polyvalent cations by CaCl_2 , CaCO_3 , NaCl addition and a right
1191 combination of ions such as NaCl + mix of Na and K or $\text{CaCl}_2+\text{MgCl}_2$;

1192 • employing some additional ingredients (different acids to control the pH, amino acids
1193 that compete with asparagine, antioxidant compounds, asparaginase, etc.).

1194 Many of the most successful reviewed intervention strategies could also be applied to other
1195 sweet and non-sweet bakery products, and the critical summary of applied studies on cookies
1196 can be useful for the industry and other research in this specific production area.

1197 Moreover, it is important to take into account that some of the AA mitigation
1198 strategies related to cookies formulation changes may have an impact on the organoleptic
1199 and nutritional properties of the final product (e.g., excessive or insufficient browning,
1200 generation of off-flavors, inadequate rising, excess sodium intake, etc.) and thus on the final
1201 quality and consumers' acceptance. The studies reported in the literature have not all
1202 thoroughly assessed the industrial feasibility point of view and not all evaluated in detail the
1203 effect of the AA mitigation strategies on the overall quality of the final product, making
1204 further research on the most promising reduction solutions necessary.

1205 **CRedit authorship contribution statement**

1206 **Maria Alessia Schouten:** Writing - Original Draft, Investigation, Visualization. **Silvia**
1207 **Tappi:** Review & Editing. **Pietro Rocculi:** Review & Editing. **Santina Romani:**
1208 Conceptualization, Writing - Review & Editing, Supervision.

1209 **Conflicts of Interest**

1210 The authors declare no conflicts of interest.

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1590 Figure 1. Number (a) of articles published per year, from 2004 to the present, on the effect
1591 of cookies' formulation on acrylamide formation with the related cumulative trend and
1592 percentage proportion (b) of each ingredient studied per year.

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