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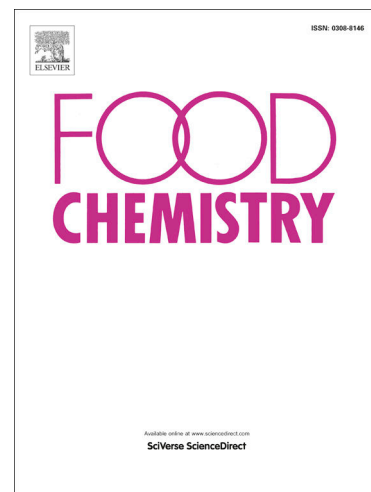
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# Mitigation of heterocyclic aromatic amines in cooked meat

## Part I: Informed selection of antioxidants based on molecular modeling

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**Abstract:**

This work aimed to develop a method permitting an informed choice of antioxidants to reduce carcinogenic heterocyclic aromatic amine (HAA) formation during proteinaceous food cooking. Therefore, a three-step approach was developed. First, the most promising antioxidants were selected using molecular modeling approaches. For this, analog design was used to highlight the most suitable antioxidants based on their diversification potential using bioisosteric replacement. Then, structure activity relationship studies allowed drawing the relevant properties for inhibiting HAA formation and explained partly the inhibitory activity. Secondly, the approved antioxidants were tested in ground beef patties to assess their inhibitory properties against HAA formation. Resveratrol was found to be the most efficient as it totally inhibited MeIQ and reduced MeIQx and PhIP formation by 40 and 70%, respectively. Finally, natural ingredients rich in these antioxidants were evaluated. Oregano was found to totally inhibit MeIQ formation and to reduce by half MeIQx and PhIP formation.

**Keywords:** Heterocyclic aromatic amines, mitigation, antioxidants, molecular modeling, analog design, Structure Activity Relationship (SAR), formulation.

**1. Abbreviations**

Heterocyclic Aromatic Amine (HAA); 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx); 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ); 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoline (MeIQ); 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx); 2-amino-1-methyl-6-phenyl-imidazo-[4,5-*b*]pyridine (PhIP); 2-amino-3,4,7,8-tetramethyl-3*H*-imidazo[4,5-*f*]quinoxaline (TriMeIQx).

50

## 51 2. Introduction

52 The preparation and cooking of meat products impact their microbial load, palatability and  
53 composition. However, some of these compositional changes such as the formation of  
54 heterocyclic aromatic amines (HAAs) have been linked to the promotion of certain cancers (e.  
55 g., colorectum, breast, prostate) in meat consumers (Zheng & Lee, 2009). Recently, the  
56 International Agency for Research on Cancer classified red meat and processed meat as  
57 probably carcinogenic (group 2A) and carcinogenic (group 1), respectively (International  
58 Agency for Research on Cancer, 2018). To reduce HAA impact on human health, mitigation  
59 strategies were proposed. Today, one of the most promising approaches is to act on the  
60 formulation of red meat products by adding natural antioxidants that limit the formation of  
61 HAAs (Meurillon & Engel, 2016). Although there is a large variety of antioxidants that can  
62 reduce HAA formation in meat (Lee et al., 2020), their choice remains mostly empirical.  
63 Indeed, the antioxidants tested for HAA inhibition are often chosen arbitrarily because they  
64 already display other medicinal properties (Tengilimoglu-Metin, et al., 2017) and are widely  
65 used in cooking in a given region. Research works often focused on a particular type of meat  
66 or on formulations based on cultural or geographical considerations such as green tea  
67 formulation for Chinese research works (Khan, et al., 2017).

68 To our knowledge, no method for making a reasoned choice exists to date and therefore, there  
69 is a real need to develop a well-reasoned mitigation strategy for choosing the best ingredients  
70 or preferred diets. The present work hypothesized that medicinal chemistry approaches could  
71 help in choosing antioxidants and in explaining their reactivity against HAA formation. A  
72 unique and original method based on medicinal chemistry knowledge was therefore  
73 developed to choose antioxidants best suited to inhibit HAA formation. This method allowed  
74 a rationalization of the choice of antioxidants, which is novel and could be subsequently  
75 transposed to other process-induced toxicants such as polycyclic aromatic hydrocarbons. In

76 the first part of the paper, an approach adapted from medicinal chemistry and widely used to  
77 choose and optimize lead compounds with the help of molecular modeling methods  
78 (Andricopulo, Salum, & Abraham, 2009; Bohacek, McMartin, & Guida, 1996) was  
79 implemented in order to select potent antioxidants for the inhibition of HAA formation. The  
80 rationale was to show which known antioxidants would be the most suitable for drug  
81 development by the implementation of analog design strategies which rested upon the  
82 improvement of existing active molecules to get rid of some side-effects or unwanted  
83 properties of the original compound. Analog design (Wermuth, 2010) permitted the discovery  
84 of direct analogs (analogs possessing structural and functional similarities), structural analogs  
85 (analogs possessing only chemical similarities but presenting different pharmacological  
86 properties) and functional analogs (chemically different compounds displaying similar  
87 pharmacological properties). Following this selection step, other molecular modeling methods  
88 were used to draw the relevant properties required for a good inhibition of HAA formation  
89 and explain their reactivity. Then, in the second part, the different antioxidants put forward by  
90 the analog design approaches were tested at different concentrations in meat samples in real  
91 conditions, using a widely consumed meat product (ground beef patties) prepared by a  
92 common cooking method (pan frying) according to WHO recommendations. In the final part,  
93 natural ingredients known to be rich in the previous antioxidants were tested under similar  
94 conditions in order to take into account complex systems more representative of common  
95 culinary practices.

96

### 97 **3. Materials and Methods**

#### 98 ***3.1. Chemicals and standards***

99 Dichloromethane, acetonitrile and methanol were organic trace analysis grade solvents  
100 (Sigma-Aldrich, France). Diatomaceous earth used for the preparation of accelerated solvent

101 extraction (ASE) cells was obtained from Sigma-Aldrich. Five HAA standards and TriMeIQx  
102 used as internal standard were obtained from Toronto Research Chemicals (North York,  
103 Canada). Resveratrol 99% and quercetin hydrate 95% were bought from Acros Organics  
104 (Geel, Belgium). Carvacrol (>98%, FC, FG) and (–)-epicatechin (>90%, HPLC) were bought  
105 from Sigma-Aldrich.

106

### 107 ***3.2. Molecular modeling and structure–activity relationship (SAR) studies***

108 A bibliographic research was performed to identify the active principles known as inhibitors  
109 of HAA formation in order to achieve molecular modeling to select the best antioxidants  
110 among them (Meurillon & Engel, 2016). A list of 17 compounds was obtained (figure 1).

111

112 To rationalize the selection of the antioxidants for further testing, analog design approaches  
113 were used. This strategy relied on the “drug-like” concept (or thereafter the “lead-like”  
114 concept) in medicinal chemistry (Lipinski, 2004). To choose between different active  
115 compounds having the same biological target, medicinal chemists are looking for their  
116 potential of structural diversification by bioisosteric replacement of functional groups, for  
117 instance. This would permit in case of synthesis problems, unwanted side-effects or even  
118 intellectual property matter to be able to develop quickly and efficiently a new drug with  
119 similar activity from the pipeline of the initial drug analogs.

120

121 *Search for structural analogs based on Tanimoto coefficient.* To find new analogs of a drug or  
122 as in the present study, antioxidants or natural active principles sharing equivalent inhibitory  
123 properties against HAA formation, the most straightforward way is to find new entities with  
124 highly similar chemical structures to that of the original compound. Binding and mode of  
125 action should be preserved between analogs and original compound. To this end, analogs

126 based on the high similarity of structural fingerprints measured by the Tanimoto coefficient  
127 (describing the ratio of the shared structural elements in respect to the total number of  
128 structural elements present in both molecules, Rogers & Tanimoto, 1960) were searched. This  
129 strategy was applied here on the 17 already proven antioxidants (Figure 1) by screening the  
130 ZINC (ZINC Is Not Commercial) database (Figure 2a) (Sterling & Irwin, 2015). The ZINC  
131 database encompasses about 230 million purchasable compounds coming from different  
132 databanks (natural products, synthetic compounds from chemical companies and Food &  
133 Drug Administration (FDA) approved drugs). The rationale behind this step was that more  
134 screened compounds (structurally similar to a studied antioxidant) would lead to more chance  
135 of discovering a potent analog (i.e. potent HAA inhibitor). The studied antioxidant with the  
136 most analogs would be therefore the most interesting from a biomolecular point of view, as it  
137 displayed a large panel of analogs with potentially the same reactivity against HAA  
138 formation. This step only relied on atomic composition and the fact that structurally similar  
139 molecules would bind to similar targets and therefore display the same biological activity. It  
140 permitted the assessment of a number of referenced compounds (in this case from databanks  
141 of natural products and FDA approved products) that presented similar structural fingerprints  
142 with the studied antioxidants and therefore determined the potential extent of such family to  
143 inhibit HAA formation. The Tanimoto index used for this search was set to 0.8, which  
144 indicated 80% of substructure homology between two molecules and then expanded to 0.7 to  
145 broaden the scope of potentially relevant molecules.

146  
147 *Functional analog search based on scaffold hopping.* To increase the diversity of active  
148 molecules with the aim to maintain the inhibitory activity while avoiding side effects, the  
149 solution was to look for functional analogues of the known antioxidants. For this purpose,  
150 scaffold hopping was used in the present work. This method consisted in the search for



151 compounds with different core structures but sharing a global 3D shape very similar to the  
152 original compound. In medicinal chemistry it allows alternative starting points for lead  
153 optimization or to generate backup candidates for advanced compounds. For this purpose, the  
154 program LigCSRre (Quintus, Sperandio, Grynberg, Petitjean, & Tuffery, 2009; Sperandio,  
155 Petitjean, & Tuffery, 2009) implemented on the RPBS (Ressource Parisienne en  
156 Bioinformatique Structurale) platform (Alland, et al., 2005) (Figure 2b) combining 3D  
157 maximal substructure search and molecular similarity was used. A databank of 2048 active  
158 compounds was screened. It permitted determination of a range of new molecules from  
159 different families potentially able to show an inhibitory effect on HAA formation. The  
160 LigCSRre software returned a list of compounds ranked by similarity scores and a specific  
161 file containing the best compounds superimposed onto the query (as to know the studied  
162 antioxidants). The similarity score rested especially upon a calculated value, the Zscore, based  
163 on the number of shared bonds and considered significant above 2. In this work, Zscore value  
164 was deliberately chosen above 2 in order to only restrict to the best results. However, for  
165 antioxidants for which the query did not return results above 3.01, the Zscore was decreased  
166 to 2 to evaluate the reply quality at this level of similarity (Supplementary Data 2).

167  
168 *Pharmacophore models by QSAR.* Having identified the most relevant antioxidants, the  
169 following step was to explain the reasons why they displayed such inhibitory activity. That  
170 implied studying chemical scaffold and associated functional groups. This approach used the  
171 pharmacophore concept. A pharmacophore is an abstract description of molecular features  
172 that are necessary for molecular recognition of a ligand by a biological macromolecule. In  
173 medicinal chemistry, the establishment of a pharmacophore model allows definition of all  
174 parts of a molecule that are necessary for its biological activity. A pharmacophore model  
175 explains how structurally diverse ligands can bind to a common receptor site. Knowing that, it

176 becomes easier to develop new analogs possessing the same targeted activity. Typical  
177 pharmacophore features include hydrophobic centroids, aromatic rings, hydrogen bond  
178 acceptors or donors, cations and anions. The features need to match different chemical groups  
179 with similar properties, in order to identify novel ligands. A well-defined pharmacophore  
180 model includes both hydrophobic volumes and hydrogen bond vectors. In computational  
181 chemistry, pharmacophores are used to define the essential features of one or more molecules  
182 with the same biological activity and serve as the starting point for developing 3D-QSAR  
183 models useful in drug discovery. In this research work, the pharmacophore model was built  
184 after superposing validated antioxidants from the same structural family using the ZINC  
185 PHARMER tool (Koes & Camacho, 2012). The model was created based on physicochemical  
186 properties of the selected antioxidants as to know hydrophobicity, aromaticity, donor or  
187 acceptor of hydrogen bonds, ionic or electrostatic bonds and used for screening the ZINC  
188 database (Figure 2c).

189  
190 *Electrostatic potential surface computation.* To increase the chance of discovering highly  
191 similar compounds, another important component was added, the electrostatic potential  
192 surface distribution. Indeed, to be able to compare different structurally similar compounds to  
193 our selected antioxidants, the electrostatic surface was representative of both the electronic  
194 distribution within aromatic cycles and partial charges of each atom. For structure-based drug  
195 design, the visual representation of electrostatic potentials provides insights into protein–  
196 ligand interactions, and hence can play a major role in elucidating the relationship between  
197 structure and reactivity of a biomolecular system. Nowadays, colorful plots of electrostatic  
198 potential are used in drug design to rationalize trends in organic reactivity and binding in  
199 host–guest complexes. In the present work, the different antioxidant molecules were modeled  
200 with Vega ZZ version 3.1 which allowed in particular charge assignment and energy

201 minimization necessary for the computation of surface electrostatic potentials. This was  
202 computed using APBS (“Adaptive Poisson-Boltzmann Solver”) plugin tool implemented in  
203 the PyMol Molecular Graphics System, Version 1.3, Schrodinger, LLC. From this calculation,  
204 the positive and negative isosurfaces were contoured at +5 and -5 kT/e, respectively (Figure  
205 2d).

206

### 207 ***3.3. Meat sample preparation***

208 Ground beef was obtained from Société Convivial (Gannat, France). The meat was certified  
209 from Charolais cattle and contained 11% fat. Stored at  $-80\text{ }^{\circ}\text{C}$ , it was thawed overnight in the  
210 fridge at  $4\text{ }^{\circ}\text{C}$  one day before use.

211 The four natural products rich in phenolic antioxidants were found at the local supermarket:  
212 ‘Extra Fine Capers’ from Maille (France), Pinot Noir Burgundy wine bottles, organic green  
213 tea in bags from Naturela and dried oregano from Ducros. They were added in two ways:  
214 marinated or blended in. For red wine and green tea samples, the meat patties were fully  
215 marinated in each solution for 2, 4 or 6 hours. For the caper and oregano samples, as well as  
216 for the four pure chemical compounds, the necessary amount of antioxidant (0.1%, 0.25% and  
217 0.5% mass concentration) was first manually ground in a stainless steel mortar and then added  
218 to meat patties and mixed four times for 20 seconds. After mixing, patties of 26 g of the same  
219 dimension were shaped with a round stainless steel ring. The patties were vacuum sealed and  
220 stored at  $-18\text{ }^{\circ}\text{C}$ .

221 Marinade durations were chosen according to literature data on marinated meat (Busquets,  
222 Puignou, Galceran, & Skog, 2006; Melo, et al., 2008) to induce a decrease in HAAs while  
223 being realistic with common household practices. Concentrations in active principles and  
224 natural ingredients were chosen to be realistic from a consumer point of view: high enough to

225 display a potential inhibitory effect based on literature data (Gibis & Weiss, 2012; Friedman,  
226 Zhu, Feinstein, & Ravishankar, 2009) but low enough to be accepted in meat recipes.  
227 For each formulation and concentration, 3 samples were prepared and further cooked and  
228 tested for HAA levels.

229

### 230 ***3.4. Cooking method***

231 To study the formation of HAAs during cooking, ground beef patties were cooked in a  
232 stainless steel frying pan (17 cm diameter) on a controlled-temperature induction hob (Bosch  
233 Electroménager, Saint-Ouen, France) according to the method described by Planche et al.  
234 (2017). A sheet of 11 µm thick aluminum foil was laid on the bottom of the frying pan to  
235 recover juice released during meat cooking. Cooking conditions were used to simulate  
236 medium meat (core 70 °C, according to WHO recommendations for ground meats).  
237 According to Planche et al. (2017), these cooking conditions corresponded to 14 min heating  
238 (turned over three times) at 200 °C at the bottom of the pan. Temperatures at the core of the  
239 meat and at the bottom of the pan were monitored by thermocouples (RS Components,  
240 Beauvais, France).

241

### 242 ***3.5. Quantification of HAAs in ground beef patties***

243 Five different HAAs (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP), likely to be found in meat,  
244 were determined by ultra-high-performance liquid chromatography–atmospheric pressure  
245 chemical ionization-tandem mass spectrometry (UHPLC–APCI-MS/MS) based on a liquid  
246 chromatography–atmospheric pressure chemical ionization-tandem mass spectrometry (LC–  
247 APCI-MS/MS) method developed for chicken meat (Chevolleau, Touzet, Jamin, Tulliez, &  
248 Debrauwer, 2007) and since adapted for analyzing beef samples (Kondjoyan, Chevolleau,  
249 Greve, et al., 2010). The choice of APCI was based on a previous work (Chevolleau, S., et al.,

250 2007) which showed that positive APCI was better suited than ESI for the detection of 10  
251 HAAs. These results were in agreement with other published works (Guy, Gremaud, Richoz,  
252 & Turesky, 2000) and were further confirmed for all the studied HAAs. However, it should be  
253 noted that several papers also describe ESI-based analytical methods for HAA analysis, since  
254 ESI is much more widespread than APCI in laboratories.

255

256 Briefly, 1 g of lyophilized ground beef sampled from the whole cooked sample was first  
257 treated with 1 M NaOH for protein denaturation. After accelerated solvent extraction (ASE)  
258 (addition of 11 g of diatomaceous earth, extraction with dichloromethane) and purification of  
259 the extract, the resulting residue was re-dissolved in 200  $\mu$ L of the starting LC mobile phase.  
260 Separation was performed on an Accela 600 LC system (Thermo Fisher Scientific, France),  
261 using a Hypersil GOLD C8 column (Thermo Fisher Scientific,  $50 \times 2.1$  mm,  $1.9 \mu$ m) with  
262 prefilter. Five microliters of the final extract were injected, and separation was performed at  
263 30 °C at a flow rate of 0.5 mL/min. Chromatographic separation was achieved using mobile  
264 phases composed of **A**: AcONH<sub>4</sub> (30 mM, pH 6) / CH<sub>3</sub>CN/CH<sub>3</sub>OH (2/1) (90/10, v/v) and **B**:  
265 AcONH<sub>4</sub> (30 mM, pH 6) / CH<sub>3</sub>CN/CH<sub>3</sub>OH (2/1) (10/90, v/v). The elution gradient was as  
266 follows: 0 min 0% **B**, 0.24 min 6% **B**, 0.98 min 22% **B**, 1.86 min 22% **B**, 2.30 min 50% **B**,  
267 2.74 min 50% **B**, 3.18 min 100% **B**, 4.21 min 100% **B**, 4.50 min 0% **B**. MS detection was  
268 performed on a TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific)  
269 using positive APCI based on two specific transitions for each HAA. Typical working parameters  
270 were as follows: discharge APCI current, 3  $\mu$ A; nebulizer temperature, 420 °C; heated transfer  
271 capillary temperature, 260 °C; sheath gas flow rate, 35 a.u.; auxiliary gas flow rate, 10 a.u. The  
272 performance of the optimized method was characterized in terms of linearity ( $r^2 > 0.98$  between 1 and  
273 500 pg/ $\mu$ L) with relative concentration residual deviation lower than 15% (20% LoQ-level) for all the  
274 concentration levels of the calibration curve), measure repeatability (CV>15%) and relative bias  
275 ( $\pm 15\%$ ), limit of detection (LoD) (0.1–0.3 pg/ $\mu$ L) and limit of quantification (LoQ) (1.0 pg/ $\mu$ L). The

276 LoD was defined as the lowest concentration of analyte that could be detected with acceptable  
277 chromatographic peak shape, with quantification and confirmation ions present with a signal-  
278 to-noise ratio (S/N) greater than 3. The LoQ was the lowest concentration that met the LoD  
279 criteria, but with a S/N of 10 and both bias% and relative standard deviation (RSD) below  
280 20%. Both the LoD and LoQ have been precisely determined for each of the HAAs. However,  
281 for convenience reasons, the LoQ was set at the value of 1 pg/ $\mu$ L, which was common to all  
282 the 5 HAAs and corresponded to the lower point of the calibration range. Since APCI may be  
283 subject to signal suppression or enhancement due to co-extracted matrix constituents,  
284 evaluation of matrix effects (ME) was carried out by comparing the calibration curves  
285 obtained by a calibration range in the solvent and in the matrix ( $n = 3$ ).  $ME (\%) = ((\text{matrix}$   
286  $\text{range slope/solvent range slope}) - 1) \times 100$ . In this context, matrix effects were interpreted as  
287 follows: negative and positive values for ME highlighted matrix-induced suppression and  
288 enhancement, respectively. Between  $-20\%$  and  $20\%$  the ME was considered small, between  $-$   
289  $50\%$  and  $-20\%$  or  $20\%$  and  $50\%$  the effect was medium, and for values of ME below  $-50\%$  or  
290 higher than  $50\%$  the effect was considered strong. The matrix effects precisely determined for  
291 each of the HAAs were less than  $20\%$  and could be considered negligible. Each cooked meat sample  
292 was extracted and analyzed in triplicate. HAA contents were expressed in nanograms of compound per  
293 gram of freeze-dried product.

294

### 295 **3.6. Data analysis**

296 Data were analyzed using Statistica software 10.0 (Statsoft, Maisons-Alfort, France). To  
297 determine whether the formulation (antioxidant or natural ingredient) had an effect on the  
298 level of HAAs in cooked meat, a one-way analysis of variance (ANOVA;  $p < 0.05$ ) was  
299 performed on data from UHPLC–APCI-MS/MS analyses. A Dunnett mean comparison test  
300 was then performed on the resulting dataset, to determine which formulation concentration  
301 was distinct from the control sample ( $p < 0.05$ ). When a significant difference was found, a

302 Duncan mean comparison test was performed on the dataset to differentiate the concentrations  
303 of a given antioxidant ( $p < 0.05$ ).

304

## 305 **4. Results and Discussion**

### 306 *4.1. Choice of relevant antioxidants by molecular modeling*

307 The first part of this study was dedicated to adapting methods used for drug design in  
308 medicinal chemistry to select promising antioxidants and explain their inhibitory activity. The  
309 rationale behind this approach was, given known inhibitors of HAAs, **1**) which ones would be  
310 retained as good drug candidates in medicinal chemistry and **2**) how to explain their inhibitory  
311 activity.

312 Figure 1 shows a list of 17 antioxidants of different families chosen for this study based on a  
313 previous review work of literature data on antioxidants known to be HAA inhibitors  
314 (Meurillon & Engel, 2016).

315 The next step relied on the “drug-like”/“lead-like” concept of medicinal chemistry. The  
316 rationale to choose antioxidants for HAA inhibition would therefore be linked to their  
317 potential diversification. This suggested that many other natural or synthetic compounds  
318 could play a similar inhibitory role than these known antioxidants. The selected strategy was  
319 based on analog design approaches consisting of modifying an existing active molecule in  
320 order to prepare a new molecule showing chemical and/or biological similarity to the original  
321 model compound (with the aim for example in the present case to limit the impact on meat  
322 organoleptic qualities and to deal with consumer acceptance issues). Thus, the more an  
323 antioxidant would display analogs, the more it would be suitable for further inhibition  
324 experiments. Indeed, an antioxidant inhibitor of HAA formation may present an aroma default  
325 in mixture with meat and therefore may not be accepted by consumers; such strategy would

326 permit discovery of other compounds as inhibitors but less problematic from an organoleptic  
327 point of view. The results from analog design approaches are presented in Table 1.

328  
329 Concerning *closely-related analogs*, Figure 1 already displayed such compounds among  
330 known inhibitors of HAA formation. Epigallocatechin and epicatechin are direct analogs as  
331 they display structural and functional similarities, as do cafestol and kahweol. However, if the  
332 success likelihood (i.e. inhibitory effect against HAAs) is relatively high using one or the  
333 other direct analog, the chance to circumvent secondary effects would be low as the molecules  
334 are similar.

335  
336 *Structural analogs* for the different antioxidants studied are displayed in Table 1. These  
337 results already showed that rutin and allicin could be discarded from this study as they  
338 displayed a poor diversification potential (i.e. no structural analogs) and therefore a poor  
339 “drug-like” potential. The detailed results obtained for all the targeted antioxidants were  
340 reported in Supplementary Data 1. As an example, the similarity search done for quercetin  
341 gave a list of 26 different referenced compounds. The variability was mostly displayed by the  
342 presence of other functional groups such as chloride-like in ZINC299830137 or hydroxyl-like  
343 in myricetin (ZINC3874317) or the replacement of one hydroxyl of quercetin by another  
344 reactive group such as sulfate-like in quercetin 3'-sulfate (ZINC14644472) or methoxy like in  
345 tamarixetin (ZINC6484604). This study evidenced that a lot of close compounds existed with  
346 little modification as the main differences lied in functional groups and substituting positions.  
347 This suggested that many other natural or synthetic compounds could play a similar inhibitory  
348 role than these known antioxidants with however the inconvenience that the 3D structure was  
349 not taken into account.

350



351 *Functional analogs* were then searched for and are also presented in Table 1. These analogs  
352 could display some equivalent functionalities (such as potentially similar inhibitory effects)  
353 with wide structural diversity. The method used was scaffold hopping which considered 3D  
354 structural similarity. Table 1 summarizes the data found for the different antioxidants studied  
355 and confirms the conclusion of the previous step, that rutin and allicin could be discarded for  
356 the rest of the study since they do not present any functional analogs. Carnosol, carnosic acid,  
357 rosmanol, cafestol, kahweol, epigallocatechin gallate, vitamin E, pyridoxamine and diallyl  
358 sulfide can also be dismissed from the study as they did not display results above 3.01 (and  
359 few above 2). Supplementary Data 2 show the detailed results obtained for all the targeted  
360 antioxidants. As an example, quercetin displayed 4 compounds with a Zscore above 3.01.  
361 From the 3D representation, it appeared that the similar active site was the bicyclic part of the  
362 molecule as the superimposition of the antioxidant and the found compounds was optimal for  
363 this part of the molecule and of less good quality for the other parts.

364  
365 Therefore, based on the similarity search and the scaffold hopping step, resveratrol, luteolin,  
366 quercetin, carvacrol, epigallocatechin and epicatechin displayed the most “drug-like” potential  
367 as they presented a large capacity for improvements given the number of structural and  
368 functional analogs they possessed.

369  
370 To determine the relevant properties for a good inhibitory potential, pharmacophore studies  
371 were realized based on the antioxidant structures the most “drug-like” (i.e. resveratrol,  
372 luteolin, quercetin, carvacrol, epigallocatechin and epicatechin). The aim of this step was to  
373 define which functional groups were necessary for antioxidants to exhibit HAA inhibitory  
374 properties, which came back to define the functional groups necessary for molecular  
375 recognition of a ligand by a biomolecule. Using dedicated software, the pharmacophore study

376 was done for quercetin and luteolin (Supplementary Data 3) with the use of different  
377 databases of bioactive products. The different properties of these two antioxidants focusing on  
378 hydrophobicity, aromaticity, donor or acceptor of hydrogen bonds, ionic or electrostatic bonds  
379 were studied by running the software with these data. This highlighted that these two  
380 antioxidants were described by the same pharmacophore model and most importantly that  
381 three hydrophobic regions and two of hydrogen bonds were the basis of this pharmacophore  
382 model (Supplementary Data 3). This was most probably a prerequisite to promote significant  
383 inhibitory activities against HAA formation.

384  
385 To try to explain the reactivity of the selected antioxidants, their electrostatic potential  
386 surfaces were computed (Supplementary Data 4). Bicyclic scaffolds of both quercetin and  
387 luteolin displayed an important positive electrostatic potential in opposition to the *meta*  
388 hydroxyl groups which were strongly electronegative. So finally, quercetin and luteolin  
389 displayed a similar representation of electrostatic field thus explaining their similar reactivity.  
390 Both quercetin and luteolin belong to the flavonoid family of polyphenolic antioxidants, are  
391 among the most prevalent in fruits and vegetables and among the most potent flavonoids from  
392 natural origin for their *in vitro* biological activity. They also displayed similar pharmacophore  
393 results and electrostatic potential representation. Therefore, to select which one will be  
394 studied in the rest of this research work, the price argument was used. Looking at Sigma-  
395 Aldrich quotation for these two active principles, it appeared that quercetin was much more  
396 available (different kinds of packaging, of purity, etc.) and more than a thousand times less  
397 expensive than luteolin for a similar purity (>95%). Quercetin was therefore selected for the  
398 rest of this research work.

399 Concerning the choice of catechin antioxidant for the next step of the study, it appeared that  
400 epicatechin was most promising as it displayed better results in term of similarity search and

401 scaffold hopping results. From a pecuniary point of view, epicatechin also remained the less  
402 expensive. Epicatechin was therefore selected for the following steps of the study.

403 The selection of carvacrol and resveratrol was less ambiguous as they were the only  
404 representatives of their analog family.

405 As the result of these molecular modeling approaches, four antioxidants were selected to be  
406 tested for the inhibition of HAA formation: carvacrol, resveratrol, (-)-epicatechin and  
407 quercetin.

408

#### 409 ***4.2. Assessment of the selected antioxidants on HAA formation inhibition***

410 The inhibitory properties against HAA formation of the four antioxidants given by the analog  
411 design approaches were assessed. For each of them, three different concentrations were tested  
412 to evaluate the dose/answer effect. Based on literature reviews (Alaejos & Afonso, 2011;  
413 Dong, Xian, Li, Bai, Zeng, 2020), this work focused on five thermic HAAs known to be  
414 found in pan fried ground beef patties: PhIP, IQ, MeIQ, MeIQx, and 4,8-DiMeIQx.

415 Table 2 shows the contribution of individual HAAs in each formulation. Control sample  
416 (ground beef patty without any antioxidant) exhibited clearly four thermic HAAs: MeIQ,  
417 MeIQx, 4,8-DiMeIQx, and PhIP, but no IQ. Their amounts were similar to those reported by  
418 other groups for pan-fried ground beef patties as summarized by Alaejos & Afonso (2011). In  
419 the present study, PhIP level in the control meat sample was found to be 1.3 ng/g of cooked  
420 meat and in literature reviews, it ranged between 0 and 32 ng/g for pan-fried ground beef  
421 patties. IQ and MeIQx levels ranged from 0 to 7–8 ng/g of cooked meat in other research  
422 studies and were found to be not detected and 4.0 ng/g, respectively, in this work. As for 4,8-  
423 DiMeIQx and MeIQ, their levels ranged from 0 to 3 ng/g based on literature review and here  
424 reached 0.4 ng/g and 0.2 ng/g of cooked meat respectively.

425 *PhIP inhibition.* Carvacrol and resveratrol were the most effective antioxidants tested here to  
426 inhibit PhIP formation. Carvacrol at 0.25% inhibited PhIP formation by 51% while resveratrol  
427 at 0.25% inhibited PhIP formation by 71%. Quercetin and epicatechin needed higher  
428 concentrations to display an inhibitory effect. At a concentration of 0.5% of epicatechin 61%  
429 inhibition was observed but none at lower concentrations (0.1 and 0.25%). Similarly, the  
430 present study showed that quercetin at 0.5% tended towards reducing by half PhIP formation  
431 (54% of inhibition). This result was in accordance with several other research works (Cheng,  
432 Chen, & Wang, 2007; Oguri, Suda, Totsuka, Sugimura, & Wakabayashi, 1998; Zhu, et al.,  
433 2016) on model systems or on beef patties and using higher concentrations of this flavonoid  
434 that induced inhibition ranging from 40 to 75% of PhIP formation. Zhu et al. (2016) found  
435 that the presence of a hydroxyl group at different positions, especially the B-ring and C-ring  
436 of the flavone skeleton, like in quercetin, significantly affected flavonoid inhibition effects on  
437 PhIP formation. Thus, they suggested that the simultaneous occurrence in quercetin of a  
438 hydroxyl group at 3'-position of B-ring and 3-position of C-ring would greatly enhance the  
439 resultant inhibition effects on PhIP formation. Salazar et al. analyzed the role of various  
440 phenolic compounds on the PhIP produced in model systems to highlight the structural  
441 characteristics that favored the inhibition of this HAA formation (Salazar, Arámbula-Villa,  
442 Hidalgo, & Zamora, 2014). Among these compounds, they studied resveratrol, epicatechin  
443 and quercetin. At a concentration of 10  $\mu\text{mol}$  of phenolic compound (equimolar with  
444 phenylalanine, the main precursor of PhIP), they found that resveratrol was a better inhibitor  
445 of PhIP formation than quercetin and that epicatechin was the weakest inhibitor among the  
446 three. The three of them have two hydroxyl groups in the *meta* position of the A-ring but the  
447 difference of activity could be explained by the two hydroxyl groups in the *ortho* position of  
448 the B-ring for quercetin and epicatechin.

449 The present results in pan-fried ground beef patties confirmed those obtained in model  
450 systems by Salazar et al. (2014), as a better inhibition of PhIP was observed for resveratrol  
451 than for quercetin and epicatechin. The similar results observed for epicatechin (61%  
452 inhibition at 0.5%) and quercetin (54% inhibition at 0.5%) in this study could be explained by  
453 their similar 3D structure (Supplementary Data 5). In the same way, as shown in  
454 Supplementary Data 6, quercetin and epicatechin displayed similar representation of their  
455 electrostatic potentials. Their bicyclic scaffold displayed an important positive electrostatic  
456 potential in opposition to the *meta* hydroxyl groups which were strongly electronegative. The  
457 hydroxyl group of resveratrol and carvacrol displayed the same electronegative potential but  
458 contrary to the other two antioxidants, their scaffolds also displayed electronegative potential.  
459 The electronegative potential around the hydroxyl group explained its reactivity with the  
460 Strecker aldehyde intermediates of HAA formation.

461 To explain this reactivity, a mechanism was proposed in Figure 3 based on the work of Cheng  
462 et al. on the inhibition of PhIP formation by reaction of epigallocatechin gallate on  
463 phenylacetaldehyde, which is the key intermediate in this HAA formation (Cheng, et al.,  
464 2009). It rested upon an electrophilic substitution of phenylacetaldehyde by the A-ring (C-6 or  
465 C-8) of quercetin, (–)-epicatechin and resveratrol. The electron-withdrawing property of the  
466 conjugated carbonyl at the C-ring of quercetin could explain its lack of reactivity with the  
467 Strecker aldehyde. The highest reactivity of resveratrol could be explained by a lower steric  
468 hindrance as well as the repartition of its electrostatic field as displayed by our modeling  
469 (Supplementary Data 4). Carvacrol, a monophenolic compound substituted by alkyl groups  
470 was by far less reactive for this kind of reaction as alkyl groups, donors by inductive effect,  
471 were less activating than hydroxyl (donor by conjugation) and therefore less *ortho/para*  
472 director. The results of inhibition tests coupled to molecular modeling and structure–activity  
473 relationship studies showed the importance of a polyphenolic scaffold with hydroxyls in the

474 *meta* position to activate the electrophilic substitution sites. From the experimental results,  
475 steric hindrance and electron-withdrawing grouping near the A-ring had a negative effect on  
476 the inhibitory properties of the antioxidants. Studies of the electrostatic field representation  
477 allowed to think that the reactive hydroxyl groups should be surrounded by negative  
478 isosurface especially around the carbon involved in the electrophilic substitution and the  
479 repartition of the electrostatic fields should be somewhat linear to promote the reaction. This  
480 fact accounted for the difference of inhibitory properties between carvacrol and resveratrol on  
481 one hand and quercetin and epicatechin on the other.

482 *MeIQx inhibition.* MeIQx was found in larger quantities ( $p < 0.01$ ) in ground beef samples as  
483 found by Ni et al. (2008). Of the four antioxidants studied, resveratrol and carvacrol were the  
484 most promising to inhibit MeIQx formation at the concentrations tested. At 0.25%, resveratrol  
485 inhibited MeIQx by 40%. Carvacrol at 0.25% permitted an inhibition of 33% of MeIQx  
486 formation. A previous study interested in antibacterial and HAA inhibitory properties of  
487 carvacrol in grilled ground beef patties described inhibition level of 72% for MeIQx  
488 (Friedman, et al., 2009) but the concentration used (1% w/w) was more than in the present  
489 study (0.1, 0.25 and 0.5% w/w).

490 *4,8-DiMeIQx inhibition.* 4,8-DiMeIQx, as MeIQx, belongs to the quinoxaline type of HAAs  
491 and is most probably formed *via* a dialkyl pyrazine radical intermediate. Therefore, one would  
492 have expected similar inhibition results for MeIQx and 4,8-DiMeIQx at least in term of  
493 antioxidants having inhibitory properties. In the present work none of the antioxidants studied  
494 displayed inhibitory activity against 4,8-DiMeIQx. These results are in accordance with other  
495 research studies, such as the work of Zeng et al. (2016) that showed no inhibition of 4,8-  
496 DiMeIQx by quercetin in a model system even at a concentration higher than in the present  
497 study. It could be thus postulated that either the levels of antioxidants employed were too low  
498 to imply an inhibitory activity against 4,8-DiMeIQx or as the amount of 4,8-DiMeIQx was

499 lower than that of MeIQx, it was difficult to follow such a small variation in quantity. *MeIQ*  
500 *inhibition*. The best results in HAA inhibition by antioxidants were obtained for MeIQ.  
501 Resveratrol even at the lowest concentration (0.1%) inhibited totally MeIQ formation.  
502 Epicatechin at a concentration of 0.25% or 0.5% achieved the same result. Concerning  
503 quercetin, a higher concentration (0.5%) was required to totally inhibit the formation of this  
504 HAA. Carvacrol was not found to inhibit MeIQ formation at the concentrations used in this  
505 study but Friedman et al. described an inhibitory effect of 58 % at a higher concentration (1%  
506 w/w) (Friedman, et al., 2009).

507 Based on the present results, resveratrol was found to be the most potent antioxidant tested as  
508 it inhibited totally the formation of MeIQ, and by 40 and 71% the formation of MeIQx and  
509 PhIP, respectively.

510

#### 511 ***4.3. Assessment of the inhibitory properties of the natural products rich in the chosen*** 512 ***antioxidants on HAA formation in cooked meat***

513 To each active principle studied was associated a natural product known to be rich in that  
514 antioxidant in order to determine the inhibitory potential against HAA formation of the  
515 antioxidant pure or in a mixture found in nature and used in household preparations. Thus in  
516 parallel to resveratrol, epicatechin, quercetin and carvacrol studies for HAA inhibition, red  
517 wine, green tea, caper and oregano formulations, respectively, were investigated. Different  
518 concentrations and marinade times were chosen to be representative of consumer's practices  
519 and high enough to display a potential inhibitory effect based on literature data (Gibis &  
520 Weiss, 2012; Friedman, Zhu, Feinstein, & Ravishankar, 2009) but low enough to be accepted  
521 in meat recipes.

522 Table 3 shows the contribution of individual HAAs in each formulation.

523 *PhIP inhibition.* Concerning natural products rich in antioxidants, oregano (rich in carvacrol)  
524 at higher concentrations (0.25 & 0.5%) had a tendency to decrease PhIP formation by more  
525 than two fold (60 and 54% inhibition, respectively). This result agreed with those described  
526 by Damašius et al. (2011) which showed a similar range of inhibitory effect of oregano on  
527 PhIP formation. The range of PhIP inhibition with oregano was also in accordance with the  
528 results found for carvacrol in the previous part of this work (51% of PhIP inhibition at a  
529 concentration of 0.25%). From the results of Quelhas et al. (2010), a better inhibition of PhIP  
530 formation was expected for green-tea-based formulations but in their work PhIP control level  
531 was higher. The difference in reactivity could be explained by the variable content in active  
532 ingredient in food matrices. Indeed, as shown by Henning et al. (2003) the content of  
533 epicatechin in green tea varied from 6.5 to 15.4 mg/100 mL among similar kinds of green tea  
534 and from 0 mg/100 mL in decaffeinated green tea to 19.7 mg/100 mL in green tea  
535 supplement. This variation could explain the differences observed from one study to another,  
536 especially since in the previous part of this study, the active principle (epicatechin) at a  
537 concentration of 0.5% displayed an inhibition of PhIP formation of 61%. From the results of  
538 Busquets et al. (Busquets, et al. 2006; Melo, et al., 2008), better inhibition results for red wine  
539 were expected but the lower inhibitory effect observed could be potentially explained by  
540 confounding factors including the meat matrix used, the red wine used (different antioxidant  
541 content depending on the wine), the duration of marinade. Indeed, as was the case for green  
542 tea, the level of resveratrol in similar wines (pinot noir) could vary from 2.3 to 10.5 mg/L  
543 according to its geographical origin (Stervbo, Vang, & Bonnesen, 2007). This explained the  
544 difference of reactivity found between resveratrol (inhibition of 71% at a concentration of  
545 0.25%) and red wine (no inhibition).

546 *MeIQx inhibition.* Poor MeIQx inhibition was observed with the natural ingredient  
547 formulations. Oregano could be the most inhibiting among the products tested as at a



548 concentration of 0.25%, it inhibited by 36% MeIQx formation. This result was similar to the  
549 inhibition found with carvacrol at 0.25% (33% inhibition). Contrary to resveratrol, red wine  
550 did not exhibit inhibitory properties against MeIQx formation, which again could be due to  
551 variation of resveratrol content among pinot noir red wines or the existence of antagonist  
552 effects with other red wine constituents. Green tea seemed to display no inhibitory effect,  
553 which was in accordance with the results found by Quelhas et al. (2010), who observed no  
554 reduction of MeIQx for marinated meat samples during 1 to 6 h in green tea. The absence of  
555 inhibition of MeIQx observed for caper and green tea was in agreement with the results found  
556 for quercetin and epicatechin, respectively, in the previous part of this study.

557 *4,8-DiMeIQx inhibition.* The different formulations studied did not inhibit significantly 4,8-  
558 DiMeIQx formation. These results were in accordance with previous studies revealing that  
559 wine (Melo, et al., 2008) and green tea (Quelhas, et al., 2010) marinades were poor inhibitors  
560 of 4,8-DiMeIQx formation in pan-fried beef. They were also in agreement with the lack of  
561 inhibition found for the different active principles in the previous part of this work.

562 *MeIQ inhibition.* Green tea and oregano were the best natural products tested in this work to  
563 inhibit MeIQ formation. The addition of 0.25% or more of oregano inhibited totally MeIQ  
564 formation. As for green tea, 2 h or 4 h marination totally inhibited MeIQ formation, while 6 h  
565 marination induced an increase of its level. The fact that there was no inhibition with red wine  
566 as opposed to resveratrol (which totally inhibited MeIQ formation at a concentration of 0.1%  
567 and more) suggested that either the concentration of resveratrol in wine was too low to  
568 achieve a good inhibition or an antagonist effect existed with other wine constituents. This  
569 antagonistic effect could be due to the large amount of phenolic acids in red wine and in  
570 particular gallic acid and caftaric acid (Van Leeuw, Kevers, Pincemail, Defraigne, &  
571 Dommes, 2014) that were both polyphenolic compounds with hydroxyl groups in *ortho*  
572 position and electron withdrawing group, strongly deactivating, in *meta* position from the

573 hydroxyl group. These phenolic acids, given their chemical structure, could also act as  
574 inhibitors of MeIQ but would be less active than resveratrol due to the presence of *ortho*  
575 deactivating group. As they were in large amounts in red wine, they could limit the inhibitory  
576 effect of resveratrol by competitive agonist reaction. In the same way, the better MeIQ  
577 inhibition of oregano than carvacrol (total inhibition vs 33% of inhibition respectively at  
578 concentration of 0.25%) could suggest that the concentrations of pure carvacrol used for the  
579 experiments were too low compare to that of oregano. Indeed as for the other natural  
580 ingredients tested, the content of active principle in oregano was highly variable. Dambolena  
581 et al. (2010) found that the relative percentage of carvacrol in oregano species varied from  
582 trace amounts to 3.57%. Another probable explanation of the difference of reactivity between  
583 oregano and carvacrol against MeIQ formation could be that other oregano constituents  
584 displayed an additive effect with carvacrol increasing the inhibition potential of MeIQ  
585 formation. This agonist effect could be explained by the high amount of thymol, an isomer of  
586 carvacrol, in common oregano species (such as *Oreganum majorana* and *Oreganum vulgare*)  
587 (Dambolena, et al., 2010). The low inhibition displayed by caper formulation could be  
588 explained by the varying amount of quercetin (from 0.03 to 1.45 mg/g depending on caper  
589 origin) and by the fact that an important part of the quercetin load in caper sample was in the  
590 form of flavonol glycosides (Inocencio, Rivera, Alcaraz, & Tomás-Barberán, 2000). The  
591 presence of sugar moieties added to the steric hindrance of the compound and could therefore  
592 explain its lack of reactivity against HAA inhibition under this form.

593

## 594 **5. Conclusion**

595 The implementation of a method based on molecular modeling allowed the reasoned choice of  
596 antioxidants inhibiting HAA formation (using analog design methods) and the explanation of  
597 their reactivity (using structure–activity relationships). The first part of this study, based on

598 medicinal chemistry approaches, highlighted the mandatory presence of a polyphenolic  
599 moiety for HAA inhibition with two hydroxyl groups in *meta* position to enhance the reaction  
600 of electrophilic aromatic substitution of the antioxidant on the Strecker aldehydes,  
601 intermediates of the Maillard reaction forming thermic HAAs. The selected antioxidants, as to  
602 know four phenolic active principles and four corresponding natural ingredients rich in these  
603 active principles, were tested in ground beef patties cooked under common household  
604 conditions. The inhibition study (*Sections 4.2 & 4.3*) highlighted that the reactivity of  
605 antioxidants, as active principles or in natural ingredients, depended on the HAA targeted. It  
606 was therefore difficult to conclude in a global manner about antioxidant capacity to inhibit  
607 HAA formation in the meat matrix. Resveratrol was shown to be the most efficient in  
608 inhibiting the HAAs studied but similar results were not found for red wine rich in this active  
609 principle. On the contrary, oregano displayed the best inhibitory properties, suggesting  
610 probable additive effect of other antioxidants present in this herb. This study pointed out the  
611 importance to consider ingredients in their entirety. Indeed, molecular modeling permitted the  
612 selection of inhibitors of HAA formation among a wide range of active principles but when  
613 looking at natural ingredients known to be rich in these antioxidants, the results were not  
614 linear. This highlighted the necessity to consider: **1)** ingredient variety, as antioxidant  
615 concentration varied from one species to another, as well as **2)** the antioxidant environment in  
616 the natural ingredient, in order to take into account potential agonist or antagonist effects on  
617 the desired inhibitory activity. In the future it would be interesting to test different varieties of  
618 a natural ingredient to take into account varietal diversity. The idea would be to see how to  
619 make a reasoned choice of the ingredient and not only of the active principle. The use of  
620 medicinal chemistry approaches developed in this study could be expanded to natural  
621 ingredient composition. It would permit consideration of the impact of other components of  
622 the natural ingredients, either agonist or antagonist, on HAA inhibition.

623 While this study gave a method adapted from medicinal chemistry to select antioxidants  
624 efficient in inhibiting HAA formation in proteinaceous food and explain their inhibitory  
625 activity, the natural ingredients rich in these antioxidants should also be tested for their  
626 organoleptic quality in meat preparations to assess consumer acceptance of these formulated  
627 products. Indeed, finding inhibitory solutions is essential but useless if the corresponding  
628 formulation is not accepted by consumers. Therefore the effects of the identified natural  
629 antioxidant addition on the meat aroma profile were assessed in a second part of this research  
630 work and compounds responsible for the differences between formulations were identified by  
631 an original approach using hedonic rating, non-verbal analysis, quantitative descriptive  
632 analysis, and gas chromatography–olfactometry. It would permit a comprehensive validation  
633 considering both safety and sensory aspects.

634

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639

## 640 **7. Conflicts of interest**

641 The authors declare that there are no conflicts of interest.

642

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770 CRediT author statement

## 771 **Mitigation of heterocyclic aromatic amines in cooked meat**

### 772 **Part I: Informed selection of antioxidants based on molecular** 773 **modeling** 774

- 775
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777 Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project  
778 administration, Funding acquisition
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- 781 **Patrick BLINET** - Investigation
- 782 **Laurent CHALOIN** - Methodology, Writing - Review & Editing
- 783 **Sylvie CHEVOLLEAU**- Investigation, Resources, Writing - Review & Editing
- 784 **Laurent DEBRAUWER** - Investigation, Resources, Writing - Review & Editing
- 785 **Erwan ENGEL**– Conceptualization, Formal analysis, Writing - Review & Editing, Validation

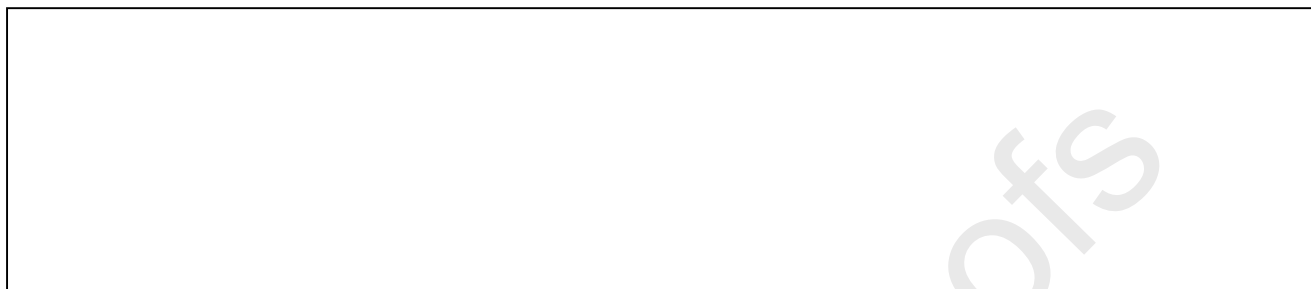
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### 788 **Declaration of interests**

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790  The authors declare that they have no known competing financial interests or personal  
791 relationships that could have appeared to influence the work reported in this paper.

792  
793  The authors declare the following financial interests/personal relationships which may be  
794 considered as potential competing interests:  
795



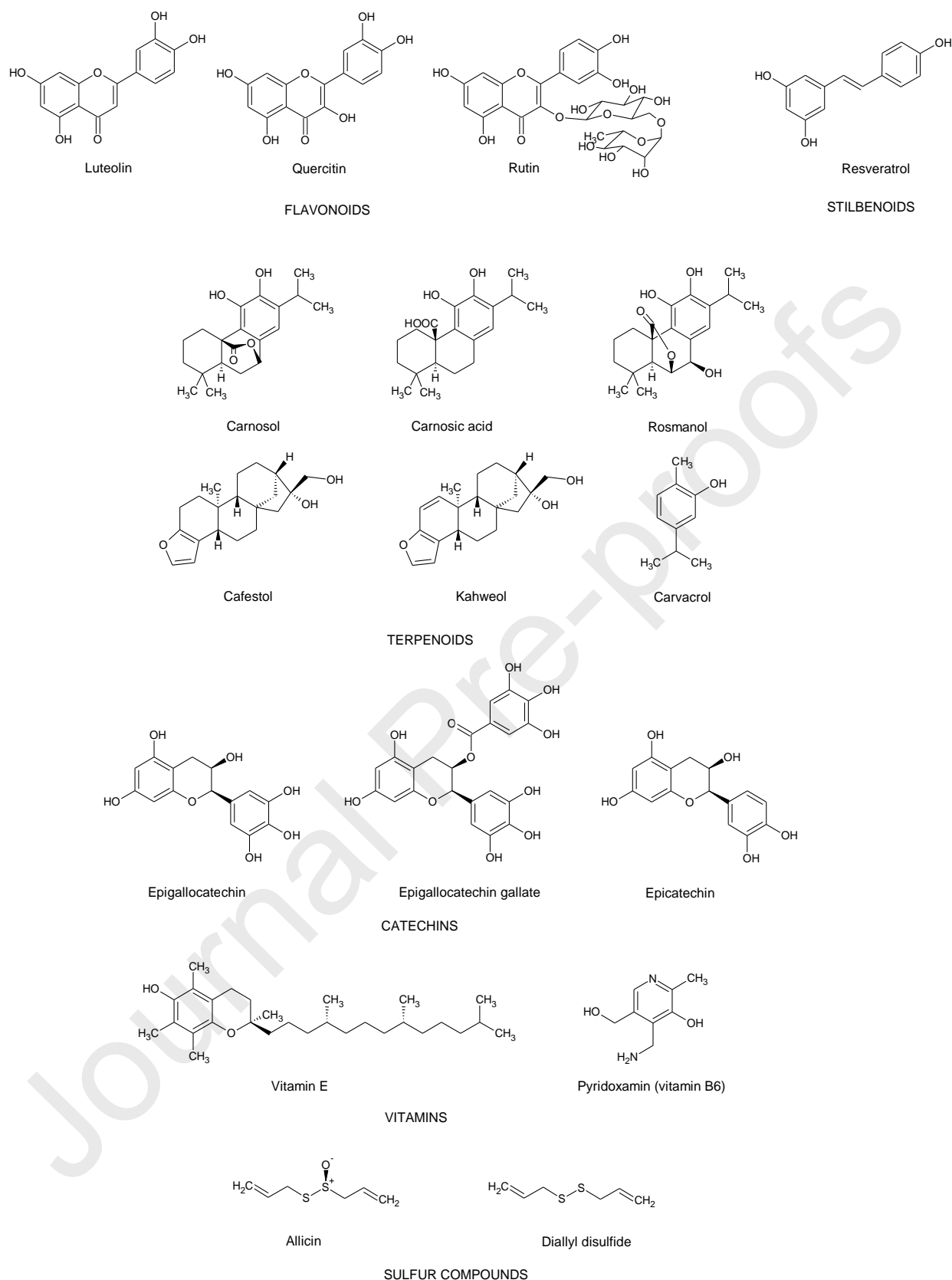
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801 **Highlights:**

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- Analog design strategy to make a reasoned choice of antioxidants for HAA inhibition
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- Determination of the structural properties required for inhibiting HAA formation
- 804
- Tests of HAA inhibition by 4 active principles and 4 natural ingredients
- 805
- Best formulations to reduce HAA formation

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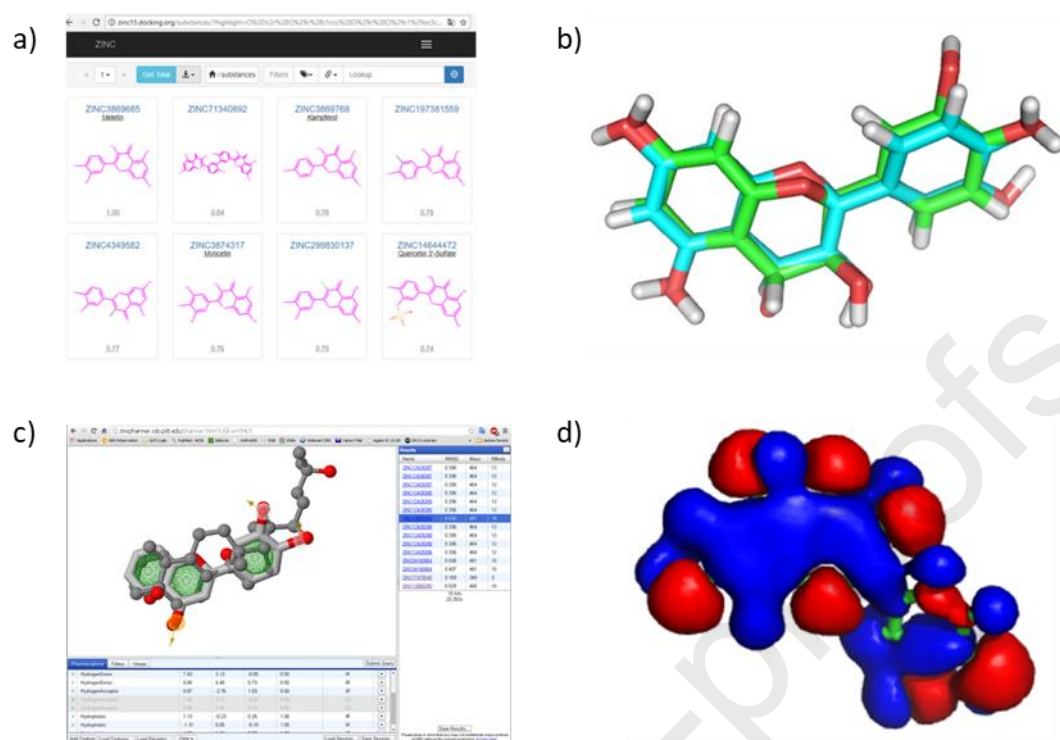




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**Figure 1.** Chemical structure of the most promising inhibitors of HAAs (Meurillon & Engel 2016)

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816 **Figure 2.** The different steps of molecular modelling and analog search based on quercetin scaffold. a)

817 *Similarity search* based on Tanimoto index (based on fingerprints defined only by the chemical formula)

818 allowing collection of all compounds having 70% similarity with quercetin, b) *Scaffold hopping* based on 3D

819 shape similarity and physicochemical properties permitting enrichment of compound libraries with similar core

820 structures, c) *Pharmacophore model* defined by the main molecular features of quercetin (aromatic cycle,

821 hydrogen bond donors and acceptors) allowing the screening of chemical databases for compounds fulfilling

822 these criteria, d) *Electrostatic potential surface determination* to help in defining organic reactivity by

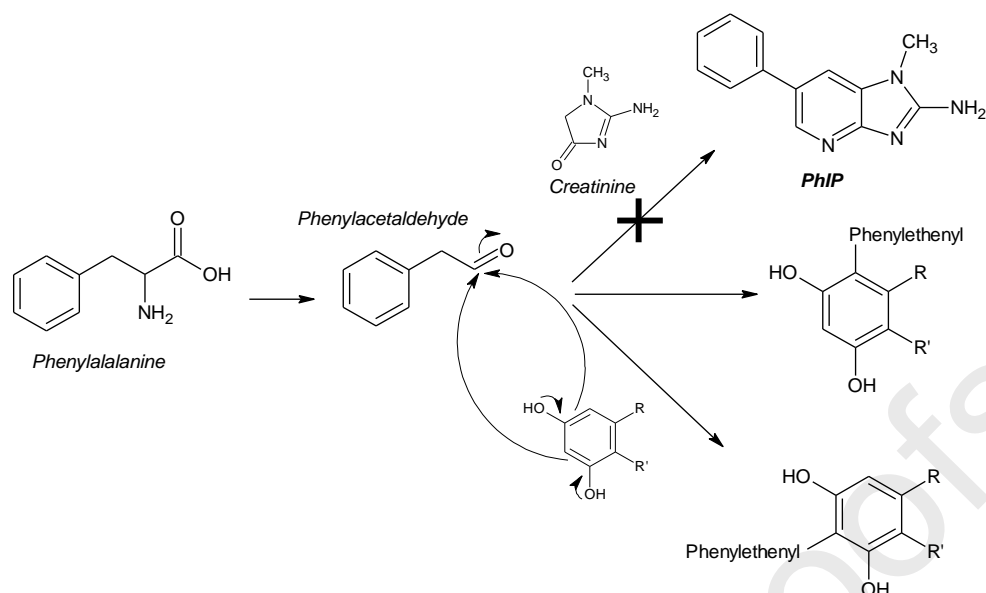
823 examining electron distribution within the chemical structure. Electrostatic potential surface was computed using

824 Adaptive Poisson-Boltzmann Solver with a probe radius of 1.4 Å. Positive (blue) and negative (red) isosurfaces

825 at  $\pm 5$  kT/e and visualized by VMD.

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 829 **Figure 3.** Postulated pathways for inhibitory activity of polyphenolic compounds on PhIP  
 830 formation based on the work of Cheng et al. (2009).

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 833 **Table 1.** Number of structural analogs displaying a Tanimoto coefficient > 0.7 and number of  
 834 functional analogs given by scaffold hopping based on 3D and physicochemical similarity for  
 835 each antioxidant described in the literature as a HAA inhibitor.

Compounds	Structural analogs <sup>a</sup>	Functional analogs <sup>b</sup>
<b>Resveratrol</b>	<b>17 different compounds</b> (47 compounds including different diastereoisomers)	<b>17 compounds</b>
<b>Luteolin</b>	<b>36 different compounds</b>	<b>11 compounds</b>
<b>Quercetin</b>	<b>26 different compounds</b>	<b>4 compounds</b>
<b>Rutin</b>	1 compound with different diastereoisomers	0 (& 0 with a Zscore >2)
<b>Carnosol</b>	<b>8 different compounds</b> (51 compounds including different diastereoisomers)	0 (& 5 with a Zscore >2)
<b>Carnosic acid</b>	<b>9 different compounds</b> (37 compounds including different diastereoisomers)	0 (& 7 with a Zscore >2)
<b>Rosmanol</b>	<b>11 different compounds</b> (84 compounds including different diastereoisomers)	0 (& 1 with a Zscore >2)
<b>Cafestol</b>	<b>6 different compounds</b> (100 compounds including different diastereoisomers)	0 (& 3 with a Zscore >2)
<b>Kahweol</b>	<b>3 different compounds</b> (57 compounds including different diastereoisomers)	0 (& 5 with a Zscore >2)
<b>Carvacrol</b>	<b>5 different compounds</b>	<b>41 compounds</b>
<b>Epigallocatechin</b>	<b>11 different compounds</b> (46 compounds including different diastereoisomers)	<b>1 compound</b>
<b>Epigallocatechin gallate</b>	<b>28 different compounds</b> (100 compounds including different diastereoisomers)	0 (& 4 with a Zscore >2)

<b>Epicatechin</b>	<b>20 different compounds</b> (82 compounds including different diastereoisomers)	<b>7 compounds</b>
<b>Vitamin E</b>	<b>14 different compounds</b> (100 compounds including different diastereoisomers)	0 (& 0 with a Zscore >2)
<b>Pyridoxamine</b>	<b>17 different compounds</b>	0 (& 26 with a Zscore >2)
<b>Allicin</b>	1 compound with different diastereoisomers	0 (& 0 with a Zscore >2)
<b>Diallyl sulfide</b>	<b>7 different compounds</b>	0 (& 1 with a Zscore >2)

836 <sup>a</sup> Structural analogs based on chemical structure displaying a Tanimoto coefficient > 0.7; <sup>b</sup> Functional analogs based on scaffold hopping  
837 results and displaying a Zscore > 3.01

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840 **Table 2.** Amount of the different HAAs found in cooked ground beef patties formulated with

841 or without antioxidants.

<b>Samples</b>	<b>IQ</b>	<b>MeIQx</b>	<b>MeIQ</b>	<b>4,8-DiMeIQx</b>	<b>PhIP</b>
Control	N.D.	7.2 ± 2.2	0.4 ± 0.1	0.7 ± 0.3	2.3 ± 0.9
Epicatechin 0.1%	N.D.	10.8 ± 1.7	0.3 ± 0.1	1.1 ± 0.2	3.1 ± 0.9
Epicatechin 0.25%	N.D.	10.6 ± 4.1	N.D.***	1.1 ± 0.4	2.3 ± 1.5
Epicatechin 0.5%	N.D.	8.9 ± 3.3	N.D.***	0.8 ± 0.3	1.0 ± 0.4
Resveratrol 0.1%	N.D.	4.9 ± 1.1*	N.D.***	0.5 ± 0.2	0.8 ± 0.2**
Resveratrol 0.25%	N.D.	4.5 ± 0.6*	N.D.***	0.5 ± 0.1	0.7 ± 0.1**
Resveratrol 0.5%	N.D.	8.2 ± 1.6	N.D.***	0.8 ± 0.2	1.9 ± 0.9
Quercetin 0.1%	N.D.	8.9 ± 2.6	0.3 ± 0.0	0.8 ± 0.3	2.4 ± 0.9
Quercetin 0.25%	N.D.	11.0 ± 6.3	0.3 ± 0.2	1.0 ± 0.5	3.1 ± 2.0
Quercetin 0.5%	N.D.	5.6 ± 0.8	N.D.	0.5 ± 0.0	1.0 ± 0.2
Carvacrol 0.1%	N.D.	6.1 ± 0.5	0.3 ± 0.1	0.6 ± 0.0	1.5 ± 0.2
Carvacrol 0.25%	N.D.	5.1 ± 0.4**	0.3 ± 0.1	0.5 ± 0.1	1.2 ± 0.1*
Carvacrol 0.5%	N.D.	6.1 ± 1.2	0.3 ± 0.1	0.6 ± 0.1	1.4 ± 0.9

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843 Values expressed in ng/g of freeze-dried product; each experiment was repeated 3 times; The detection and quantification limits (expressed  
844 in ng/g of freeze-dried product) were, respectively, 0.02 and 0.2 for PhIP, IQ and 4,8-DiMeIQx and 0.05 and 0.2 for MeIQx and MeIQ; N.D.:  
845 not detected; \*: significant difference to control value,  $p < 0.05$ ; \*\*: significant difference to control value,  $p < 0.01$ ; \*\*\*: significant  
846 difference to control value,  $p < 0.001$ .

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850 **Table 3.** Amount of the different HAAs found in cooked ground beef patties formulated with

851 or without natural ingredients.

<b>Samples</b>	<b>IQ</b>	<b>MeIQx</b>	<b>MeIQ</b>	<b>4,8-DiMeIQx</b>	<b>PhIP</b>
Control	N.D.	7.2 ± 2.2	0.4 ± 0.1	0.7 ± 0.3	2.3 ± 0.9
Green Tea 2 h	N.D.	6.2 ± 1.7	N.D.***	0.6 ± 0.0	2.1 ± 0.5
Green Tea 4 h	N.D.	4.9 ± 0.7	N.D.***	0.6 ± 0.1	1.7 ± 0.9
Green Tea 6 h	N.D.	5.9 ± 2.2	0.3 ± 0.0	0.6 ± 0.2	2.1 ± 1.5
Red Wine 2 h	N.D.	7.1 ± 2.1	0.3 ± 0.0	0.8 ± 0.2	1.6 ± 0.7
Red Wine 4 h	N.D.	8.4 ± 2.8	0.4 ± 0.0	1.0 ± 0.3	1.6 ± 0.7

Red Wine 6 h	N.D.	8.2 ± 1.4	0.3 ± 0.1	1.0 ± 0.1	1.7 ± 0.6
Caper 0.1%	N.D.	5.6 ± 1.2	0.3 ± 0.0	0.6 ± 0.1	1.2 ± 0.5
Caper 0.25%	N.D.	7.0 ± 3.7	0.2 ± 0.1	0.7 ± 0.4	2.2 ± 1.5
Caper 0.5%	N.D.	7.1 ± 2.4	0.2 ± 0.1	0.7 ± 0.2	1.8 ± 0.6
Oregano 0.1%	N.D.	9.2 ± 3.2	0.5 ± 0.1	0.8 ± 0.2	3.0 ± 1.4
Oregano 0.25%	N.D.	4.6 ± 0.3	N.D.***	0.5 ± 0.1	1.0 ± 0.2
Oregano 0.5%	N.D.	5.7 ± 0.9	N.D.***	0.6 ± 0.1	1.1 ± 0.3

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Values expressed in ng/g of freeze-dried product; each experiment was repeated 3 times; Red wine is rich in resveratrol, green tea in epicatechin, caper in quercetin and oregano in carvacrol; The detection and quantification limits (expressed in ng/g of freeze-dried product) were, respectively, 0.02 and 0.2 for PhIP, IQ and 4,8-DiMeIQx and 0.05 and 0.2 for MeIQx and MeIQ; N.D.: not detected; \*: significant difference to control value,  $p < 0.05$ ; \*\*: significant difference to control value,  $p < 0.01$ ; \*\*\*: significant difference to control value,  $p < 0.001$ .