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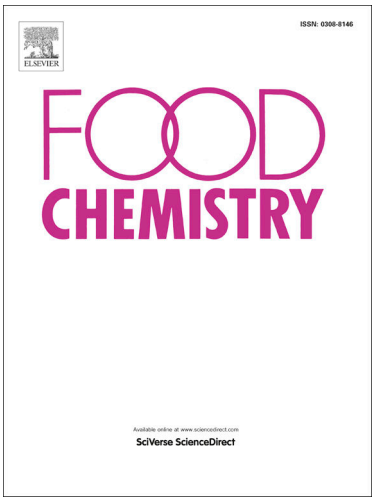

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Mitigation of heterocyclic aromatic amines in cooked meat

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).

2. Introduction

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

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

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

3. Materials and Methods

3.1. Chemicals and standards

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

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

3.2. Molecular modeling and structure–-activity relationship (SAR) studies

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

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

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

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

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

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

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

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

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

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

3.3. Meat sample preparation

Ground beef was obtained from Société Convivial (Gannat, France). The meat was certified from Charolais cattle and contained 11% fat. Stored at –80 °C, it was thawed overnight in the fridge at 4 °C one day before use.

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

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

display a potential inhibitory effect based on literature data (Gibis & Weiss, 2012; Friedman, Zhu, Feinstein, & Ravishankar, 2009) but low enough to be accepted in meat recipes.

For each formulation and concentration, 3 samples were prepared and further cooked and tested for HAA levels.

3.4. Cooking method

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

3.5. Quantification of HAAs in ground beef patties

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

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

Briefly, 1 g of lyophilized ground beef sampled from the whole cooked sample was first treated with 1 M NaOH for protein denaturation. After accelerated solvent extraction (ASE) (addition of 11 g of diatomaceous earth, extraction with dichloromethane) and purification of the extract, the resulting residue was re-dissolved in 200 µL of the starting LC mobile phase. Separation was performed on an Accela 600 LC system (Thermo Fisher Scientific, France), using a Hypersil GOLD C8 column (Thermo Fisher Scientific, 50 × 2.1 mm, 1.9 µm) with prefilter. Five microliters of the final extract were injected, and separation was performed at 30 °C at a flow rate of 0.5 mL/min. Chromatographic separation was achieved using mobile phases composed of **A**: $AcONH_4$ (30 mM, pH 6) / CH_3CN/CH_3OH (2/1) (90/10, v/v) and **B**: $AcONH_4$ (30 mM, pH 6) / CH_3CN/CH_3OH (2/1) (10/90, v/v). The elution gradient was as 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**, 2.74 min 50% **B**, 3.18 min 100% **B**, 4.21 min 100% **B**, 4.50 min 0% B. MS detection was performed on a TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific) using positive APCI based on two specific transitions for each HAA. Typical working parameters were as follows: discharge APCI current, 3 µA; nebulizer temperature, 420 °C; heated transfer capillary temperature, 260 °C; sheath gas flow rate, 35 a.u.; auxiliary gas flow rate, 10 a.u. The performance of the optimized method was characterized in terms of linearity (r^2 > 0.98 between 1 and 500 pg/µL) with relative concentration residual deviation lower than 15% (20% LoQ-level) for all the concentration levels of the calibration curve), measure repeatability (CV>15%) and relative bias (±15%), limit of detection (LoD) (0.1–0.3 pg/µL) and limit of quantification (LoQ) (1.0 pg/µL). The

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

3.6. Data analysis

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

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

4. Results and Discussion

4.1. Choice of relevant antioxidants by molecular modeling

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

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

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

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

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

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

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

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

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

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

To try to explain the reactivity of the selected antioxidants, their electrostatic potential surfaces were computed (Supplementary Data 4). Bicyclic scaffolds of both quercetin and luteolin displayed an important positive electrostatic potential in opposition to the *meta* hydroxyl groups which were strongly electronegative. So finally, quercetin and luteolin displayed a similar representation of electrostatic field thus explaining their similar reactivity.

Both quercetin and luteolin belong to the flavonoid family of polyphenolic antioxidants, are among the most prevalent in fruits and vegetables and among the most potent flavonoids from natural origin for their *in vitro* biological activity. They also displayed similar pharmacophore results and electrostatic potential representation. Therefore, to select which one will be studied in the rest of this research work, the price argument was used. Looking at Sigma-Aldrich quotation for these two active principles, it appeared that quercetin was much more available (different kinds of packaging, of purity, etc.) and more than a thousand times less expensive than luteolin for a similar purity (>95%). Quercetin was therefore selected for the rest of this research work.

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

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

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

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

4.2. Assessment of the selected antioxidants on HAA formation inhibition

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

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

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

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

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

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

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

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

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

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

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

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

MeIQx inhibition. Poor MeIQx inhibition was observed with the natural ingredient 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 nott 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

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

5. Conclusion

The implementation of a method based on molecular modeling allowed the reasoned choice of antioxidants inhibiting HAA formation (using analog design methods) and the explanation of 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.

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

6. Acknowledgements

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7. Conflicts of interest

The authors declare that there are no conflicts of interest.

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CRediT author statement

Mitigation of heterocyclic aromatic amines in cooked meat

Part I: Informed selection of antioxidants based on molecular modeling

Maïa MEURILLON - Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition

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Laurent CHALOIN - Methodology, Writing - Review & Editing

Sylvie CHEVOLLEAU- Investigation, Resources, Writing - Review & Editing

Laurent DEBRAUWER - Investigation, Resources, Writing - Review & Editing

Erwan ENGEL – Conceptualization, Formal analysis, Writing - Review & Editing, Validation

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights:

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

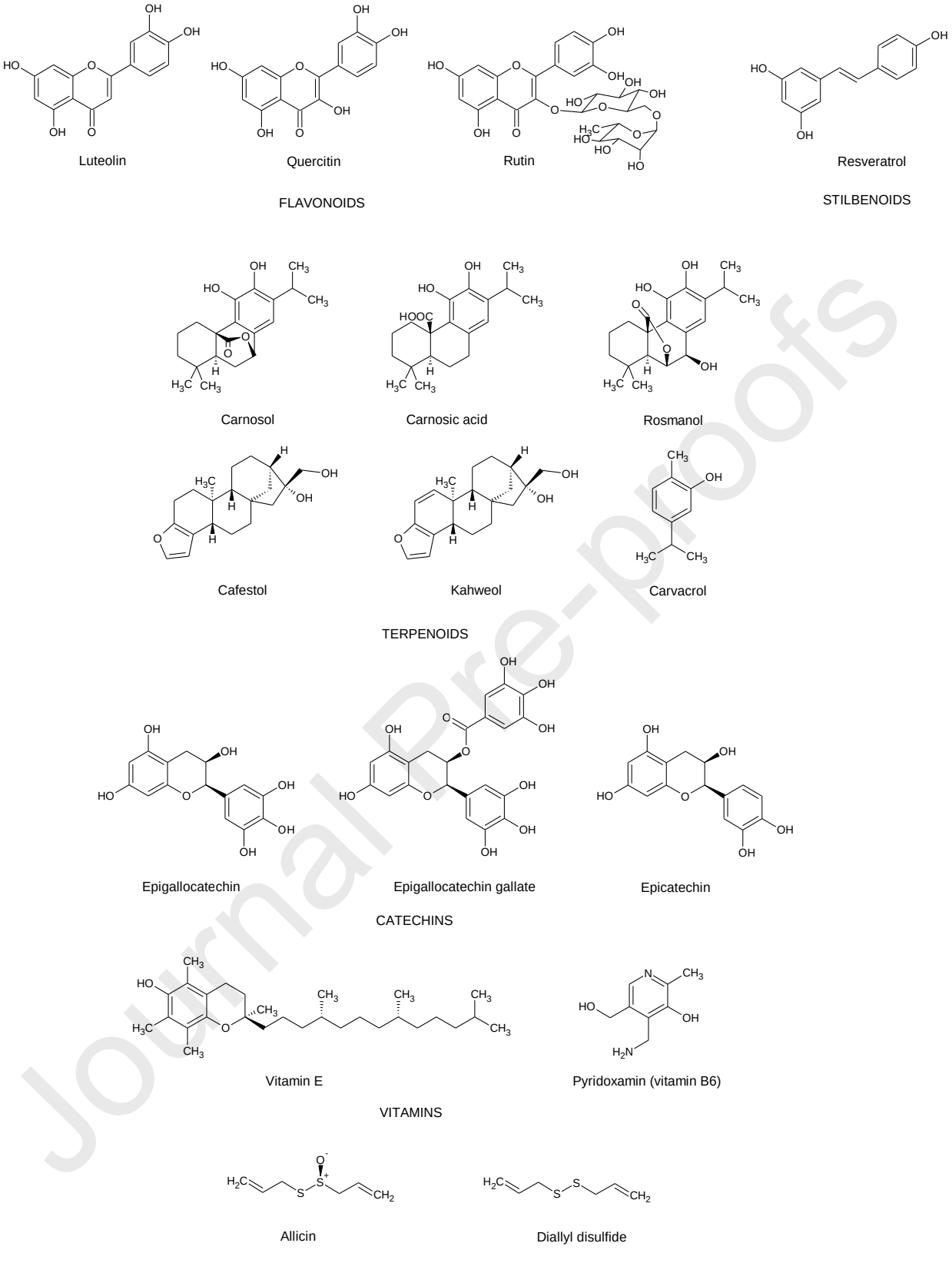


Figure 1. Chemical structure of the most promising inhibitors of HAAs (Meurillon & Engel 2016)

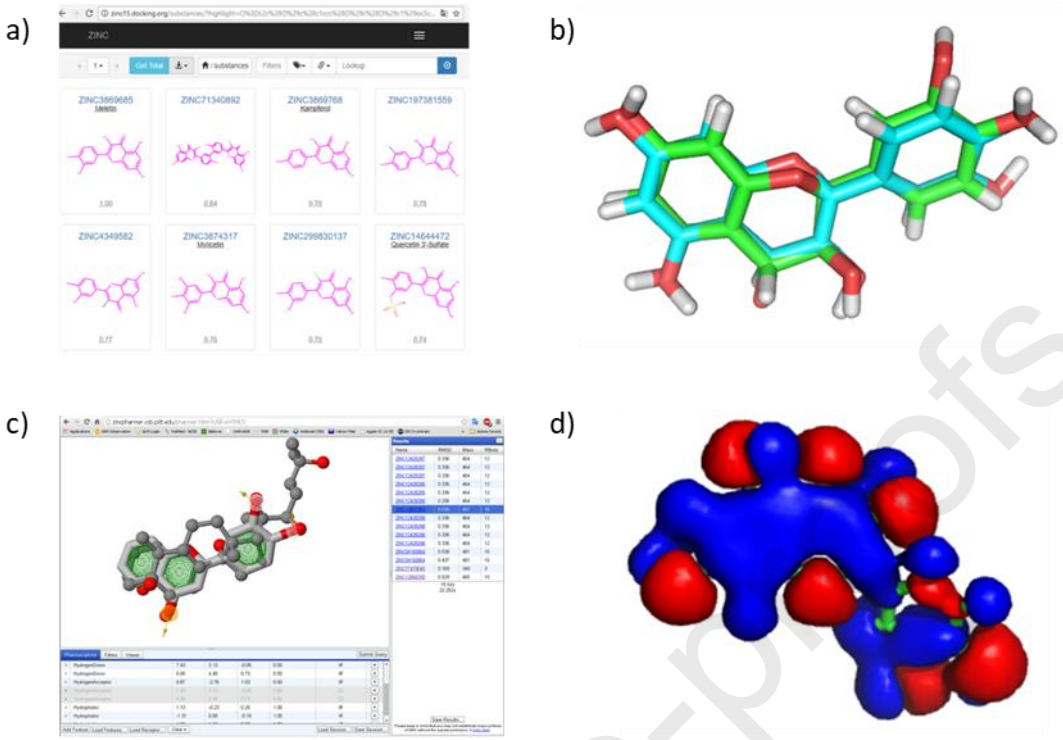
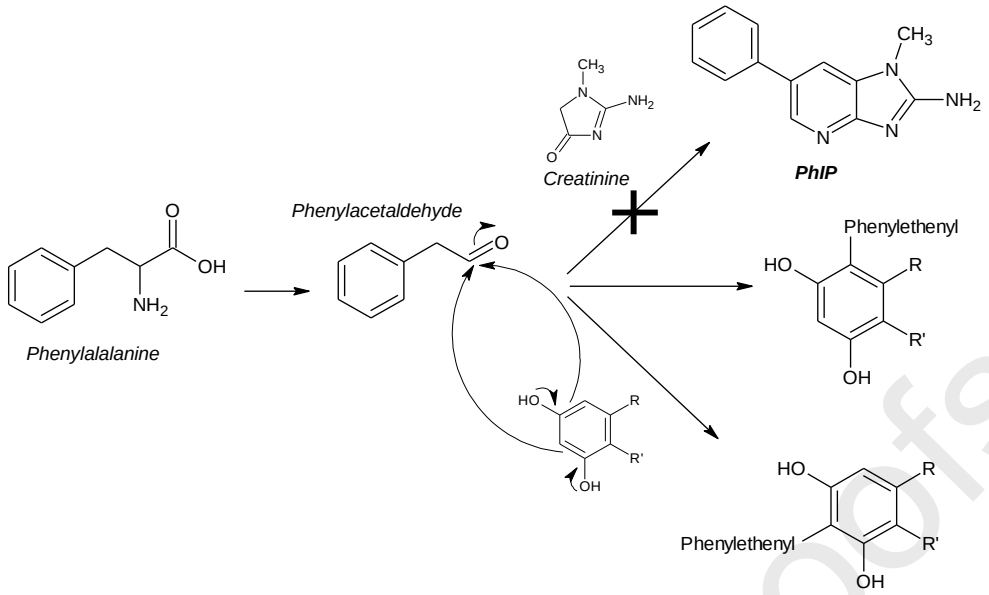


Figure 2. The different steps of molecular modelling and analog search based on quercetin scaffold. a) ***Similarity search*** based on Tanimoto index (based on fingerprints defined only by the chemical formula) allowing collection of all compounds having 70% similarity with quercetin, b) ***Scaffold hopping*** based on 3D shape similarity and physicochemical properties permitting enrichment of compound libraries with similar core structures, c) ***Pharmacophore model*** defined by the main molecular features of quercetin (aromatic cycle, hydrogen bond donors and acceptors) allowing the screening of chemical databases for compounds fulfilling these criteria, d) ***Electrostatic potential surface determination*** to help in defining organic reactivity by examining electron distribution within the chemical structure. Electrostatic potential surface was computed using Adaptive Poisson-Boltzmann Solver with a probe radius of 1.4 Å. Positive (blue) and negative (red) isosurfaces at ± 5 kT/e and visualized by VMD.



828

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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832

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

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

[a] Structural analogs based on chemical structure displaying a Tanimoto coefficient > 0.7; [b] Functional analogs based on scaffold hopping results and displaying a Zscore > 3.01

Table 2. Amount of the different HAAs found in cooked ground beef patties formulated with or without antioxidants.

Samples	IQ	MeIQx	MeIQ	4,8-DiMeIQx	PhIP
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

Values expressed in ng/g of freeze-dried product; each experiment was repeated 3 times; 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, $p < 0.001$.

Table 3. Amount of the different HAAs found in cooked ground beef patties formulated with or without natural ingredients.

Samples	IQ	MeIQx	MeIQ	4,8-DiMeIQx	PhIP
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

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, $p < 0.001$.