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Cyclization of reactive carbonyls in foods upon processing: An origin for the formation of pyridine-derived flavours and heterocyclic aromatic amines

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1. INTRODUCTION

1.1. Food processing and its consequences

Foods can be consumed in a raw state, but, most frequently, they are processed both to improve their quality and to destroy deleterious components. Food processing can be carried out in many ways. Thus, milling, cooling/freezing, smoking, heating, canning, fermentation, drying, or extrusion cooking, among others, are common industrial processes used in food manufacturing (Augustin *et al.*, 2016; Stoica *et al.*, 2013). These processes have benefits, such as destruction of food-borne microbes and toxins, improving bioavailability of nutrients, extending shelf life, or enhancing sensory characteristics and functional properties (Boekel *et al.*, 2010; Nawrot *et al.*, 1999). On the other hand, food processing can also have detrimental consequences for the involved foods (**Table 1**). Among them, nutrient degradation (Elmore *et al.*, 2010), production of deleterious sensory active compounds (Starowicz & Zieliński, 2019), or production of compounds with mutagenic or carcinogenic properties (Jaeger *et al.*, 2010) have been described.

Table 1. Detrimental effects of thermal processing.

Food constituents	Effect of heating	Examples of produced compounds
Carbohydrates	Decomposition	Carbonyl compounds
	Formation of carbonyl-amine adducts, including polymers	Advanced glycation end products (AGEs)
Lipids	Oxidation	Lipid hydroperoxides
	Hydroperoxide decomposition	Carbonyl compounds
	Formation of carbonyl-amine adducts, including polymers	Advanced lipoxidation end products (ALEs)
Amino acids/ Proteins/ Creatine	Decomposition	Carbonyl compounds
	Formation of carbonyl-amine adducts	AGEs/ALEs
	Reaction products with creatine	Heterocyclic aromatic amines (HAAs)
Phenolics	Oxidation	Quinones

This Ph.D. thesis will study the generation of two families of products formed as a consequence of food processing: flavours with the structure of pyridine and heterocyclic aromatic amines (HAAs), in an attempt to find out the chemical basis of the formation of both kinds of compounds. This knowledge should allow to promote their formation when they are desirable and to understand how their formation can be inhibited when they act as food toxicants. Thus, pyridines are potent odorants that contribute to the sensory properties of some foods (Maga, 1981). On the other hand, the formation of heterocyclic aromatic amines (HAAs) has been related to the claimed mutagenicity and carcinogenicity for processed meats (International Agency for Research on Cancer, 2018).

1.2. Pyridines

The pyridines are a large group of N-heterocyclic compounds. The simplest pyridine derivative has the same name of the group, pyridine. Its structure is related to benzene but one methine group (=CH-) is replaced by a nitrogen atom. Other pyridines are more complex because of the presence of substituents in the aromatic ring.

1.2.1. Pyridines in foods

The presence of alkylpyridines in foods has been investigated by diverse authors (Ferretti & Flanagan, 1971; MacLeod & Coppock, 1976; Watanabe & Sato, 1971; Yajima *et al.*, 1979).

The importance of heterocyclic compounds in food flavours is well-known. However, relatively little attention has been given to pyridine derivatives, even though many of them have been found in a large number of foods, specially alkylpyridines (Suyama & Adachi, 1980). In addition, hydroxypyridines also contribute to the sensory properties of some foods. However, contrarily to these alkylpyridines, hydroxypyridines are less frequently found in foods (Elhalis *et al.*, 2021).

The food systems in which pyridines have been found include food systems containing high fat and low water contents, and submitted to roasting or frying. Therefore, pyridines have been hypothesized to be produced as a consequence of the interaction between primary amines in proteins or amino acids with alkadienals (Kim *et al.*, 1996).

Foods in which pyridines have been found include meat (beef, chicken, and lamb), fish, vegetables (beans, asparagus, potatoes, and tomatoes), cereals (rice and corn), bread, nut products (almond, peanuts, and pecan), soya bean, milk and dairy products (cheese), fruits, spices and condiments (pepper), honey, coffee, and alcoholic beverages (Bicas & Rodriguez-Amaya, 2021; Maga, 1981).

1.2.2. Role of pyridines in food flavours

Pyridines are considered to contribute to food flavour to a lower extent than other heterocyclic compounds such as pyrazines. However, they provide foods with bitter, astringent, and roasted notes, among others. The flavour properties of some selected pyridines are collected in **Table 2**.

Table 2. Flavour properties produced by pyridine derivatives.

Pyridine	Flavour properties
2-Methylpyridine	Astringent and hazelnut
3-Methylpyridine	Green, earthy, and hazelnut
2-Ethylpyridine	Buttery, green, and caramel
2,5-Dimethylpyridine	Roasted, green, and earthy
2,6-Dimethylpyridine	Green
5-Ethyl-2-methylpyridine	Fatty and green

Elaborated from Maga, (1981)

1.2.3. The formation of pyridines

The formation of pyridines in foods is mostly unknown, although Suyama & Adachi (1980) suggested that they were produced by reaction of lipid-derived reactive carbonyls with amino compounds. In addition, (Kim & Ho, 1998b) suggested that the cyclization of 2,4-alkadienals was the origin of 2-alkylpyridines. A mechanism for this conversion has been suggested by Zamora *et al.*(2009). The reaction takes place as indicated in **Figure 1**. The first step is the formation of corresponding imine. This imine evolves into a cyclic derivate (the corresponding pyridine) at the same time that the amino acid residue is eliminated in the form of its vinylogous derivative (acrylamide in **Figure 1**). This pathway was developed for a possible conversion of 3-aminopropionamide into acrylamide. However, it can also be produced with ammonia or any ammonia-producing compound.

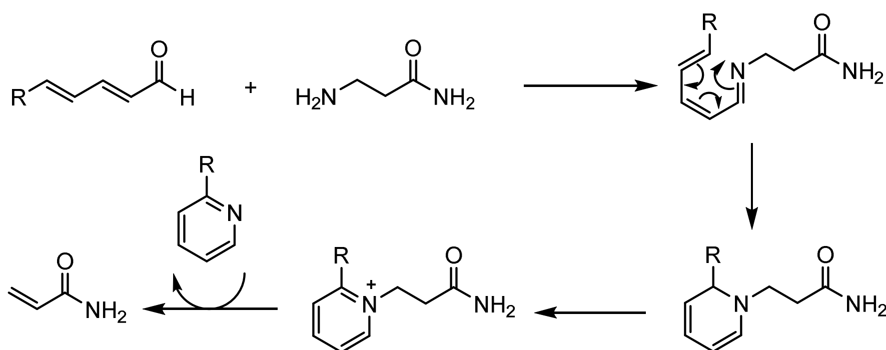


Figure 1. Pyridine formation through carbonyl-amine reaction in presence of reactive carbonyls (Zamora *et al.*, 2009).

However, the origin of other pyridines is not known and, with the exception of 2,4-alkadienals, other lipid-derived reactive carbonyls have not been related to the formation of pyridines. Nevertheless, the few examples described above suggest that pyridines can be produced by cyclization of lipid-derived carbonyl compounds.

1.3. Heterocyclic aromatic amines

Different to pyridines, heterocyclic aromatic amines (HAAs) have been the objective of numerous studies. They were one of the first groups of carcinogens isolated and identified in foods. Their isolation and identification was a consequence of the mutagenic activity observed in cooked meat and fish by Sugimura's group (Sugimura *et al.*, 2004). Heterocyclic aromatic amines are now a large group of chemical compounds which are shown in **Figure 2**.

As can be observed in the figure, their structures usually contain from 2 to 5 (generally 3) condensed aromatic cycles with one (or more) nitrogen atom(s) in their ring system and, usually, one primary amine group, with the exception of harman, and norharman. Their formations occur as a consequence of the thermal processing at high temperatures of proteinaceous foods. Since their discovery, more than thirty heterocyclic aromatic amines have been identified in cooked meat (Sugimura et al., 2004).

These compounds are usually classified into different groups depending on their formation temperature, the attributed formation procedure, or their polarity (Jägerstad et al., 1991; Zamora & Hidalgo, 2015):

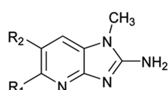
- Depending on their formation temperature, heterocyclic aromatic amines can be classified into thermic and pyrolytic heterocyclic aromatic amines. Thermic heterocyclic aromatic amines (also known as IQ- and IQx-type) have the structure of aminoimidazoazarene. They are produced in proteinaceous foods at temperatures typical of cooking/frying (~200 °C). On the other hand, pyrolytic heterocyclic aromatic amines, also known as non-IQ-type, are formed by pyrolysis of amino acids and proteins at temperatures higher than 250 °C.
- According to the attributed formation pathways, they are considered to be produced via Maillard reaction (such as the thermic heterocyclic aromatic

amines) or via pyrolytic reactions (such as the pyrolytic heterocyclic aromatic amines).

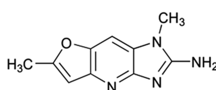
- Based on their polarity, these compounds have been classified into polar and nonpolar heterocyclic aromatic amines.

AMINOIMIDAZOAZARENES

imidazopyridine derivatives

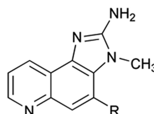


DMIP: R_1 = H, R_2 = CH_3
1,5,6-TMIP: R_1 = R_2 = CH_3
PhIP: R_1 = H, R_2 = phenyl
4'-OH-PhIP: R_1 = H, R_2 = 4'-OH-phenyl

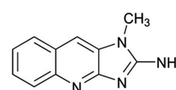


IFP

imidazoquinoline derivatives

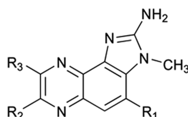


IQ: R = H
MeIQ: R = CH_3

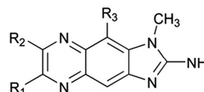


IQ[4,5-b]

imidazoquinoxaline derivatives



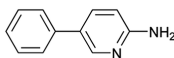
IQx: R_1 = R_2 = R_3 = H
4-MeIQx: R_1 = CH_3, R_2 = R_3 = H
7-MeIQx: R_1 = R_3 = H; R_2 = CH_3,
8-MeIQx: R_1 = R_2 = H; R_3 = CH_3,
4,8-DiMeIQx: R_1 = R_3 = CH_3, R_2 = H
7,8-DiMeIQx: R_1 = H; R_2 = R_3 = CH_3,
4,7,8-TriMeIQx: R_1 = R_2 = R_3 = CH_3
4-CH_2OH-8-MeIQx: R_1 = CH_2OH, R_2 = H, R_3 = CH_3



IgQx: R_1 = R_2 = R_3 = H
6-MeIgQx: R_1 = CH_3, R_2 = R_3 = H
7-MeIgQx: R_1 = R_3 = H, R_2 = CH_3
6,7-DiMeIgQx: R_1 = R_2 = CH_3, R_3 = H
7,9-DiMeIgQx: R_1 = H, R_2 = R_3 = CH_3

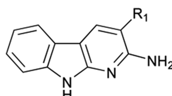
PYROLYTIC HETEROCYCLIC AROMATIC AMINES

phenylpyridine derivatives



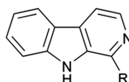
Phe-P-1

α-carbolines



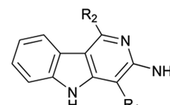
AαC: R_1 = H
MeAαC: R_1 = CH_3

β-carbolines



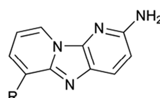
Norharman: R_1 = H
Harman: R_1 = CH_3

γ-carbolines

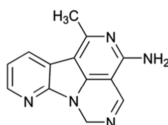


Trp-P-1: R_1 = R_2 = CH_3
Trp-P-2: R_1 = H, R_2 = CH_3

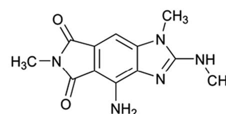
other derivatives



Glu-P-1: R = CH_3
Glu-P-2: R = H



Orn-P-1



Cre-P-1

Figure 2. Chemical structures of the main heterocyclic aromatic amines (Zamora & Hidalgo, 2015).

1.4. Thermic heterocyclic aromatic amines or aminoimidazoazarenes

Because the formation of most pyrolytic heterocyclic aromatic amines need very high temperatures, these heterocyclic aromatic amines are produced to a much lower extent than aminoimidazoazarenes under standard cooking conditions (Meurillon & Engel, 2016). Therefore, the major carcinogenic heterocyclic aromatic amines commonly found in foods have the structure of aminoimidazoazarene. Although harman and norharman are found to a significant extent in foods, they are not carcinogenic (Hagiwara *et al.*, 1992; Kawamori *et al.*, 2004).

1.4.1. Toxicological aspects

The International Agency for Research on Cancer (IARC) has classified four of these aminoimidazoazarenes into two groups: Group 2a (the compound is probably carcinogenic to humans) or group 2b (the compound is possibly carcinogenic to humans). Thus, 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) has been classified within the group 2a, and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinolone (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, it appears as 8-MeIQx in **Figure 2**), and 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) have been classified into group 2b (International Agency for Research on Cancer, 2018). Moreover, in accordance with the Ames test (*Salmonella typhimurium* reverse mutation assay), MeIQ, IQ, and MeIQx are potent mutagens (Sugimura *et al.*, 2004). This test is a bacterial short-term test for identification of carcinogens using mutagenicity in bacteria as an endpoint (Hengstler & Oesch, 2001).

Toxicological aspects of these compounds have gained importance in recent years because of the classification by IARC of processed meat into group 1 (the agent is carcinogenic to humans), and the classification of red meat into group 2a (International Agency for Research on Cancer, 2018). The reason for including heterocyclic aromatic amines among the chemical compounds suspicious of

contributing to meat carcinogenicity is double: Their presence in muscle foods when cooked, and the identification of some of these compounds as suspicious of producing cancer in human beings (Zamora & Hidalgo, 2015). However, the consumption of the amount produced of these compounds cannot be fully responsible for the carcinogenicity observed for processed meat. Therefore, a high-fat intake, and the presence/formation of other compounds in meats such as polycyclic aromatic hydrocarbons or nitrosamines are also likely contributing to the meat carcinogenicity (Ferguson, 2010).

1.4.2. Aminoimidazoazarenes commonly found in foods

Heterocyclic aromatic amines are commonly detected in heated animal-derived foods like beef, pork, chicken, and fish due to their high content of creatine, which is needed for heterocyclic aromatic amine formation (Jägerstad *et al.*, 1991). PhIP is one of the most frequently found heterocyclic aromatic amines, whereas IQ, MeIQ, MeIQx, 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx or DiMeIQx), and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) are also commonly present in processed meat. In addition to proteinaceous foods, heterocyclic aromatic amines have been isolated from other foods like coffee (Herraiz, 2002) and alcoholic beverages (Manabe *et al.*, 1993), and from environmental sources, including cooking fumes (Vainiotalo *et al.*, 1993), cigarette smoke (Smith *et al.*, 2004), and river and rain water (Ohe, 1997; Ono *et al.*, 2000).

As observed in **Table 3**, heterocyclic aromatic amine formation is dependent upon the type of proteinaceous food and the type, temperature, and length of cooking. Levels of heterocyclic aromatic amines are low or non-detectable in foods fried at 150 ºC, but a sharp increase is detected at cooking temperatures higher than 190 ºC (Johansson *et al.*, 1995). Therefore, to find significant concentrations of aminoimidazoazarenes in proteinaceous foods, a cooking temperature in the range 180-220 ºC is usually required. Furthermore, if cooking is prolonged at high temperature for a long time period, consumers can be

exposed to high amounts of heterocyclic aromatic amines that might play a major role in the increasing occurrence of pathologies such as cancers or neurodegenerative diseases (Meurillon *et al.*, 2018; Turesky, 2007).

Table 3. Presence of heterocyclic aromatic amines with the structure of aminoimidazoazarene in cooked foods.

Food	Cooking method	HAAs	Reference
Beef	Fried	IQ, MeIQ, MeIQx, DiMeIQx, PhIP	(Balogh *et al.*, 2000)
	Grilled	MeIQx, PhIP	(Gross *et al.*, 1993)
	Deep grilled	IQ, MeIQ, MeIQx, 4,8-DiMeIQx	
Bacon	Fried	MeIQx, PhIP	(Gibis *et al.*, 2015)
	Grilled	MeIQx, 4,8-DiMeIQx, PhIP	(Gross *et al.*, 1993)
Chicken	Pan fried	IQ, MeIQx, 4,8-DiMeIQx, PhIP	(Liao *et al.*, 2010)
	Deep fried	MeIQx, 4,8-DiMeIQx, PhIP	
	Charcoal grilled	MeIQx, 4,8-DiMeIQx, PhIP	
	Roasted	PhIP	
Duck	Pan fried	IQ, MeIQx, 4,8-DiMeIQx, PhIP	(Liao *et al.*, 2010)
	Deep fried	MeIQx, 4,8-DiMeIQx, PhIP	
	Charcoal grilled	IQ, MeIQx, 4,8-DiMeIQx, PhIP	
Salmon	Microwave	MeIQx	(Oz *et al.*, 2010)
	Oven	MeIQx	
	Hot plate	MeIQx	
	Barbecued	IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP	
Pork loin	Oven, hot air fried or deep oil fried.	PhIP	(Yang *et al.*, 2017)

Table 3 (cont.)

Food	Cooking method	HAAs	Reference
Chicken burger	Pan fried	IQx	(Haskaraca et al., 2016)
Hamburger patties	Fried -	IQ, MeIQ, MeIQx MeIQx	(Aeenehvand *et al.*, 2016)
Beer	-	PhIP	(S Manabe *et al.*, 1992)
Wine	-	IQ, MeIQ, MeIQx, 4,7,8-TriMeIQx	(Richling *et al.*, 1997)
Soup cubes	-	IQ, MeIQx, 4,8-DiMeIQx	(Krach & Sontag, 2000)
Soft cheese	-	MeIQx	(Gonzalo-Lumbreras *et al.*, 2010)

Elaborated from Barzegar *et al.*(2019). Abbreviation: HAAs, heterocyclic aromatic amines.

1.4.3. Estimation of aminoimidazoazarene intake in the general population

An estimate of population-average dietary intake of thermic heterocyclic aromatic amines is complex because the daily intake of them is different in each study. Thus, there are differences in study designs, and, also, on portion size, population, cooking conditions, intake frequency, etc. According to the obtained results in the different studies, heterocyclic aromatic amine intakes vary from < 2 to > 25 $ng \cdot kg^{-1}$ of body weight per day (Augustsson *et al.*, 1997; Keating & Bogen, 2001).

According to the study of Bogen & Keating (2001) (**Table 4**), heterocyclic aromatic amine intake depends on age, sex, socioeconomic factors, race-ethnicity, type of food eaten, and cooking method. Thus, in decreased order of consumption, PhIP represents over 66% of the total heterocyclic aromatic amine considered, while MeIQx and AαC are 15%, and DiMeIQx and IQ each 2% of this total input. Estimated heterocyclic aromatic amine consumption takes into account race-ethnicity, age group, and sex, and it is about 1.5- to 2-fold greater

for African Americans and Asian/Pacific Islanders than for whites. In general, female vs. male heterocyclic aromatic amine specific intakes show small differences for each heterocyclic aromatic amine within each age and racial-ethnic group. An exception is that estimated intake by male African American children is over 30-50% greater than that of females (Bogen & Keating, 2001).

Table 4. Estimated mean heterocyclic aromatic amine (HAA) intakes by race-ethnicity, age group, and sex parameters.

Race / ethnicity	**Age**	**Sex**	**Estimated HAA intake (ng/kg/day)**			
			PhIP	**MeIQx**	**DiMeQx**	**IQ**
White	Child	M/F	6.10/6.50	1.20/1.20	0.23/0.24	0.16/0.14
	Young	M/F	5.50/5.60	1.10/0.96	0.20/0.19	0.12/0.13
	Older	M/F	5.60/5.30	1.10/1.00	0.18/0.17	0.20/0.20
African American	Child	M/F	12.00/8.90	1.80/1.50	0.51/0.33	0.24/0.11
	Young	M/F	8.30/7.30	1.30/1.20	0.35/0.27	0.09/0.12
	Older	M/F	8.10/7.40	1.60/1.20	0.30/0.23	0.16/0.23
Asian / Pacific Islander	Child	M/F	10.00/9.20	1.90/1.90	0.27/0.22	0.29/0.90
	Young	M/F	5.80/5.90	1.00/1.10	0.22/0.16	0.33/0.20
	Older	M/F	8.20/9.60	1.70/1.90	0.20/0.21	0.69/1.20

Elaborated from Bogen & Keating, (2001). Abbreviations: M, masculine. F, feminine. Child <16 years, young (16–29 years), and older (>30 years).

1.5. Formation of aminoimidazoazarenes

The heterocyclic aromatic amines with the structure of aminoimidazoazarene have an imidazole ring fused to either a pyridine or a pyrazine ring. Because of their similar structures (**Figure 2**), they are supposed to be produced by means of analogous reaction pathways, although their formation mechanisms are still unclear.

Preliminary studies suggested that heterocyclic aromatic amines were produced as a result of complex reactions that involved creati(ni)ne, free amino

acids, and carbohydrates at high temperatures through Maillard reaction. Two hypothetical pathways were considered according to the Maillard reaction intermediates: A carbonyl pathway and a free radical pathway.

The first proposed pathway was a consequence of the ability of Maillard reaction to produce pyrazines, and, to a lower extent, also pyridines. These heterocyclic compounds, which are produced as a consequence of the reaction between amino acids and reducing sugars, can later react with creati(ni)ne, and aldehydes, to produce the different heterocyclic aromatic amines (**Figure 3**) (Milic *et al.*, 1993).

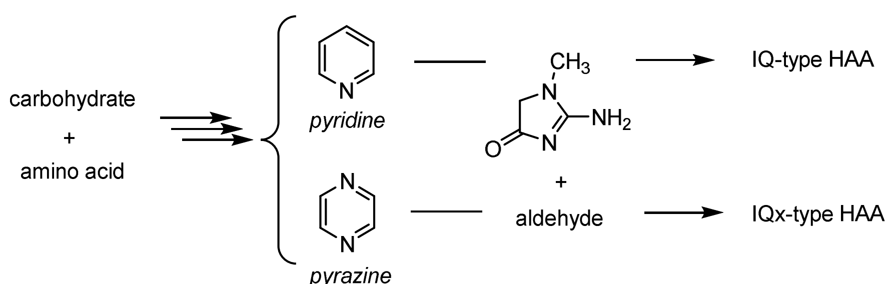


Figure 3. Proposed carbonyl pathway for heterocyclic aromatic amines formation (Zamora & Hidalgo, 2020).

Milic et al. (1993) firstly studied the pyrazine or pyridine formation by reaction of *D*-(+)-glucose and amino acids to produce 2,5-dimethylpyrazine or 2-methylpyridine. Then, the later reaction of these pyrazine or pyridine with creati(ni)ne and acetaldehyde produced MeIQx and MeIQ, respectively. However, employed reaction conditions are not representative of reactions occurring in real food systems.

Some other studies have also pointed out that radicals, especially pyridine and pyrazine radicals might also be involved in the formation of aminoimidazoazarenes (**Figure 4**). Two different pathways for this free radical formation are proposed. One involves pyridine radical and other pyrazine radical, which would react with creati(ni)ne and the corresponding aldehyde. The

reaction would finish with the formation of IQ-type and IQx-type mutagens respectively. Nonetheless, this mechanism remains relatively controversial because it has missing steps (Murkovic, 2004).

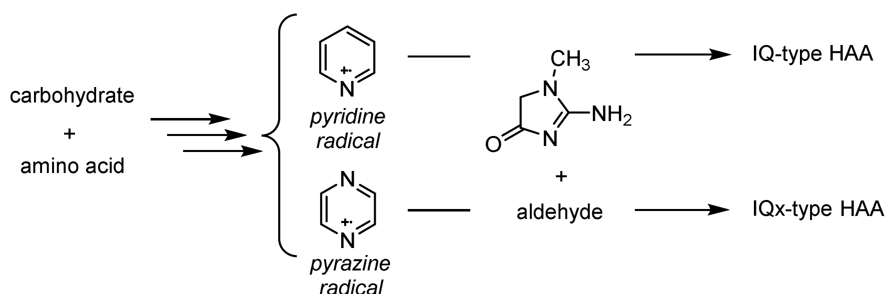


Figure 4. Proposed free radical pathway for heterocyclic aromatic amines formation (Zamora & Hidalgo, 2020).

At present, only PhIP formation pathway has been clarified. As described below, it does not follow any of the two previously proposed formation mechanisms.

Proposal of a reaction pathway for PhIP has been a long process. It was initiated by Shioya *et al.* (1987). These authors found that PhIP was produced in mixtures of creatinine, phenylalanine, and glucose. Later, Manabe *et al.* (1992) described that the reaction could also be produced in the presence of aldehydes. Some years later, Murkovic *et al.* (1999) demonstrated that phenylacetaldehyde was an intermediate in its formation. In fact, this group isolated the aldol condensation product between phenylacetaldehyde and creati(ni)ne (Zöchling & Murkovic, 2002). The formation pathway was completed some years ago by Zamora *et al*. (2014). The complete reaction pathway is shown in **Figure 5.**

The reaction is initiated by the degradation of phenylalanine to produce phenylacetaldehyde. This degradation, also known as Strecker degradation, occurs when amino acids are heated in the presence of reactive carbonyls (Zamora *et al.*, 2007). Once this aldehyde has been produced, it suffers the addition of creati(ni)ne to form the corresponding aldol product, which is later

dehydrated. The pyridine ring of PhIP is completed by successive additions of ammonia and formaldehyde. Ammonia and formaldehyde are produced by degradation of phenylalanine, phenylacetaldehyde, and/or creati(ni)ne. Additionally, formaldehyde and ammonia can react to produce formamide, which might also be an intermediate to close the pyridine ring (Zamora *et al.*, 2014).

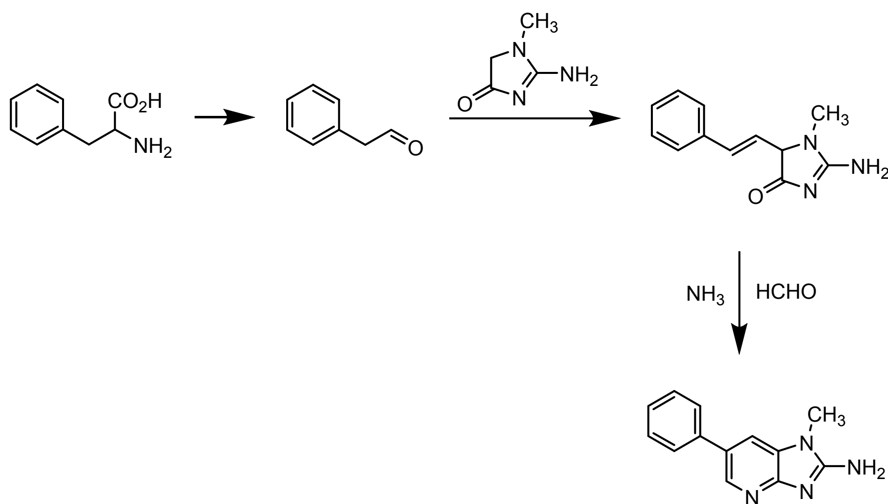


Figure 5. Proposed pathway for PhIP formation (Zamora *et al.*, 2014).

Analogously to PhIP, other heterocyclic aromatic amines with the structure of aminoimidazoazarene might also be produced similarly. Thus, reactive carbonyls other than phenylacetaldehyde would be produced and they would react with creati(ni)ne to produce the main skeleton of the different heterocyclic aromatic amines. Full structures might be later completed by means of ammonia and/or short chain aldehydes. As will be discussed below, this hypothesis, which has not been investigated so far, will be the main objective of this thesis. This hypothesis implies both that the reactive carbonyls required for the formation of the different heterocyclic aromatic amines should be produced in foods and that these reactive carbonyls should have a tendency to oligomerize and to produce cyclic/heterocyclic structures in the presence of creati(ni)ne. This tendency would also be the origin of the pyridines found in foods. Therefore, if this

hypothesis is confirmed, the formation of these kinds of compounds (some of which are desirable, but other are well-known food toxicants) can be understood and their formation potentially controlled based on this knowledge. The antecedents for the formation of reactive carbonyls in foods and their tendency to produce cyclic derivatives will be discussed in the next subsections.

1.6. Reactive carbonyls in foods: Formation of the food carbonylome

Reactive carbonyls are continuously produced in foods and the most reactive of these compounds rapidly disappear. This group of compounds is therefore in a continuous change. The whole group of carbonyl compounds present in food at a certain time has been named “food carbonylome” (Zamora & Hidalgo, 2020). Its origin is quite diverse and, frequently, the same reactive carbonyl can have different precursors. Thus, the carbonyl compounds present in foods are produced from carbohydrates, lipids, amino acids, and phenolic compounds, among other sources.

1.6.1. Reactive carbonyls produced from carbohydrates

1.6.1.1. Carbohydrates

In nature, carbohydrates are abundant components which are present in plant and animal tissues as well as in microorganisms. They are important nutrients that serve as energy source, fuel, and building materials. In addition, these compounds are often used as food additives, including anticaking agents, bulking agents, emulsifiers, gelling agents, humectants, stabilizers, sweeteners, or thickeners (Soga & Serwe, 2000; Voragen, 1998).

Some examples of carbohydrates present in foods and foods products are described in **Table 5** (Eggleston *et al.*, 2018).

Table 5. Carbohydrates present in foods.

	Food	Carbohydrates		
		Monosaccharide	**Disaccharide**	**Polysaccharide**
Fruit	Apple	Glucose, fructose and mannose	Sucrose	Starch and cellulose
	Grape	Glucose, fructose and mannose	Sucrose	Cellulose
	Strawberry	Glucose, fructose and mannose	Sucrose	Cellulose
Vegetables	Carrot	Glucose and fructose	Sucrose	Starch and cellulose
	Onion	Glucose and fructose	Sucrose	Cellulose
	Peanuts	-	Sucrose	Cellulose
	Potato	-	-	Starch and cellulose
Others	Sugar cane	Glucose and fructose	Sucrose	-
	Honey	Glucose and fructose	Sucrose	-
	Meat	Glucose	-	Glycogen
	Milk	-	Lactose	-

Carbohydrates (**Figure 6**) can be classified into different groups. Thus, they are classified:

- Into monosaccharides, oligosaccharides, and polysaccharides, according to their chemical structure. Monosaccharides are polyhydroxy-aldehydes (aldoses) or -ketones (ketoses), generally with an unbranched C-chain. When these monosaccharides polymerize in a number under 10 carbohydrate units, oligosaccharides are produced. The main oligosaccharides are the disaccharides, such as maltose or lactose. Finally, when the number of carbohydrate units are higher than 10, polysaccharides are produced. Examples are starch, cellulose, and pectin (Belitz *et al.*, 2009).

- Into simple and complex carbohydrates according to the facility of absorption and digestion (Wong, 2018).

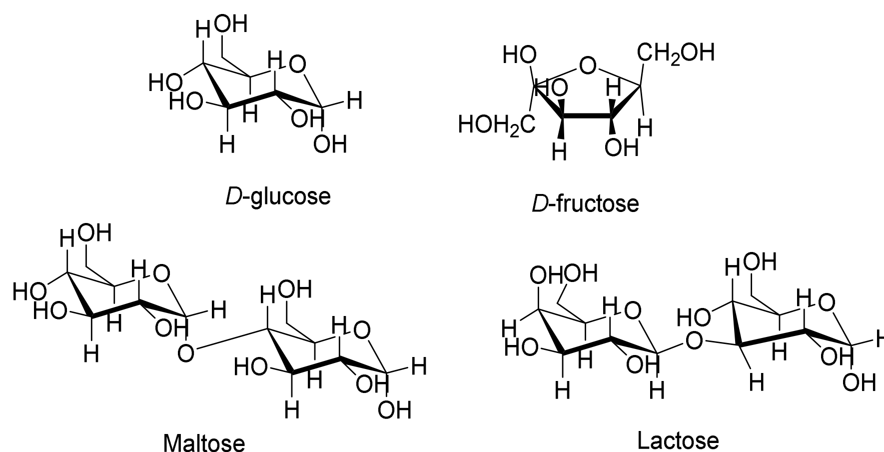


Figure 6. Structures of some carbohydrates.

1.6.1.2. Maillard reaction

Maillard reaction, also known as non-enzymatic browning, is one of the main chemical reactions that take place in foods. This reaction occurs as a consequence of food cooking, preservation, and processing, and many carbohydrates-derived reactive carbonyls are produced, which will be later involved in carbonyl-amine reactions. The carbohydrates that take part in Maillard reaction are reducing sugars, such as *D*-glucose, *D*-fructose, maltose, or lactose (**Figure 6**).

The first references to this reaction were described by Louis-Camille Maillard in 1912. He observed that, when mixtures of amino acids and sugars were heated, browning was produced. However, it would not be until the 1950s, when the first stable products of the Maillard reaction in foods (glycosylamines or Amadori products) were identified. In 1953, John E. Hodge pointed out Amadori rearrangement with a key role in the reaction and divided the reaction into three parts: First, the formation of the Amadori products, followed by their

degradation, as a second step, and, then, the formation of melanoidins (Hellwig & Henle, 2014).

A simplified scheme of Maillard reaction is shown in **Figure 7.** The pathway is divided into 3 main steps (Amaya-Farfan & Rodriguez-Amaya, 2021):

- Initial stage begins with a sugar-amine condensation. Amino acids or amines, which are nucleophilic compounds, react with the carbonyl function of reducing carbohydrates. Then, imines (*N*-substituted glycosylamine)/Schiff bases are produced. Lately, these imines undergo a process named Amadori rearrangement to produce the corresponding Amadori (1-amino-1-deoxyketose) or Heyns (2-amino-2-deoxyaldose) compounds when the initial reducing sugar is either an aldose or a ketose, respectively.
- Intermediate stage occurs when these Amadori compounds are degraded into 1-, 3-, and 4-deoxyosone compounds by means of different reactions including enolization, deamination, dehydration, cyclization, retroaldolization, isomerization, and fragmentation. These reactions occur under severe heating or prolonged storage. In addition to deoxyosones, other carbonyl compounds, furan derivatives, and other intermediates are also produced.
- Final stage comprises aldol condensations, carbonyl-amine reactions, and the formation of heterocyclic derivatives with an atom of nitrogen. In addition, polymerisation reactions are also produced and the formation of coloured products with high molecular mass (melanoidins) is a common consequence of Maillard reaction. These pigments are responsible for the colour development observed in foodstuff during thermal processes (e.g. bread, biscuit, and meat). Polymerisation reactions also contribute to the hardening of cooked and stored food (Belitz et al., 2009; Rannou et al., 2016).

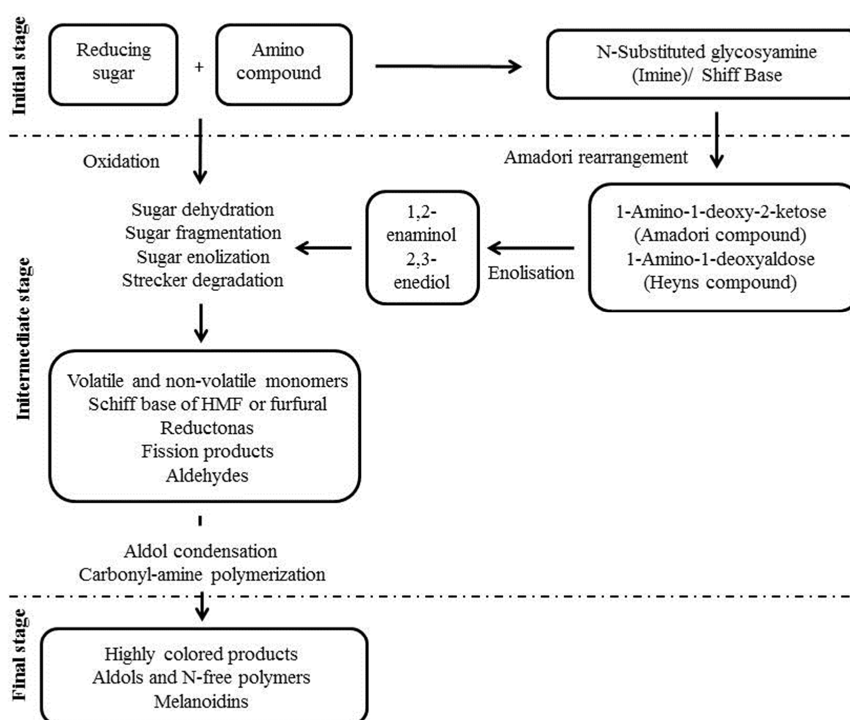


Figure 7. A simplified scheme of Maillard reaction (adapted from Purlis, 2010; Zamora & Hidalgo, 2005).

1.6.1.3. Formation of reactive carbonyls from carbohydrates

Maillard reaction produces many compounds, including the formation of aldehydes, alcohols, thiols, sulphides, acids, lactones, and a large variety of heterocyclic compounds. The formed products depend on the intermediate that is degraded. Thus, 3-hydroxy-5-hydroxymethyl-2-methyl-(5*H*)-furan-4-one, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol), or acetylformoin are produced by degradation of 3-deoxyosones, furfural is produced as a consequence of 1-deoxyosone degradation, and 2-hydroxyacetylfuran is produced by degradation of 4-deoxyosones (Zheng *et al.*, 2019). Others secondary products of Maillard reaction are reductones, such as acetylformoin and aldehydes from the reactions between deoxyosones and amino acids. This last reaction is usually known as

Strecker degradation and the produced aldehydes are known as Strecker aldehydes (Zheng *et al.*, 2019).

The most abundant carbohydrate-derived reactive dicarbonyls present in foods are 3-deoxyglucosone, 3-deoxygalactosone, and glucosone, which predominate over methylglyoxal, glyoxal, and 3,4-dideoxyglucosone-3-ene (**Figure 8**) (Amaya-Farfan & Rodriguez-Amaya, 2021).

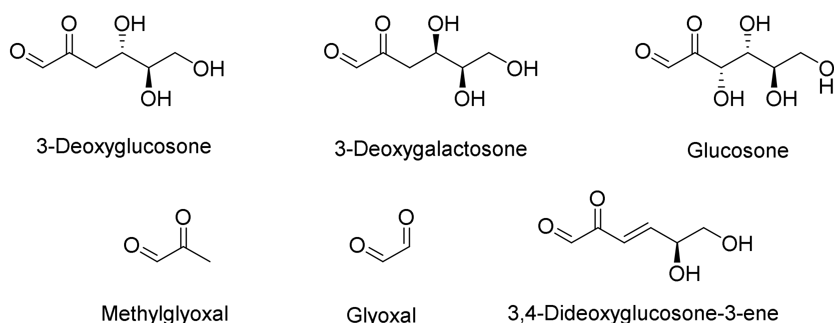


Figure 8. Carbohydrate-derived reactive carbonyls found in food.

A reaction pathway for the formation of most of these compounds is shown in **Figure 9**. As can be observed, once the Amadori compound is produced, it suffers enolizations to form glucosone and deoxyosones. Retroaldol reactions of these last compounds are the origin of glyoxal and methylglyoxal (Hellwig *et al.*, 2018).

One important product of Maillard reaction, which is produced to a significant extent in many foods, is 5-hydroxymethylfurfural (HMF). Its formation is shown in **Figure 9**. As observed in the figure, HMF seems to be a product of the intramolecular cyclization of the 3,4-dideoxyglucosone-3-ene (3,4-DGE), which is formed from 3-deoxyglucosone (3-DG) (Aktağ & Gökmen, 2021; Hellwig *et al.*, 2018). Other compounds with a structure of 2-oxofurans, such as furfural and 2-acetylfurfan are also formed as a consequence of carbohydrate degradation (Kanzler *et al.*, 2017).

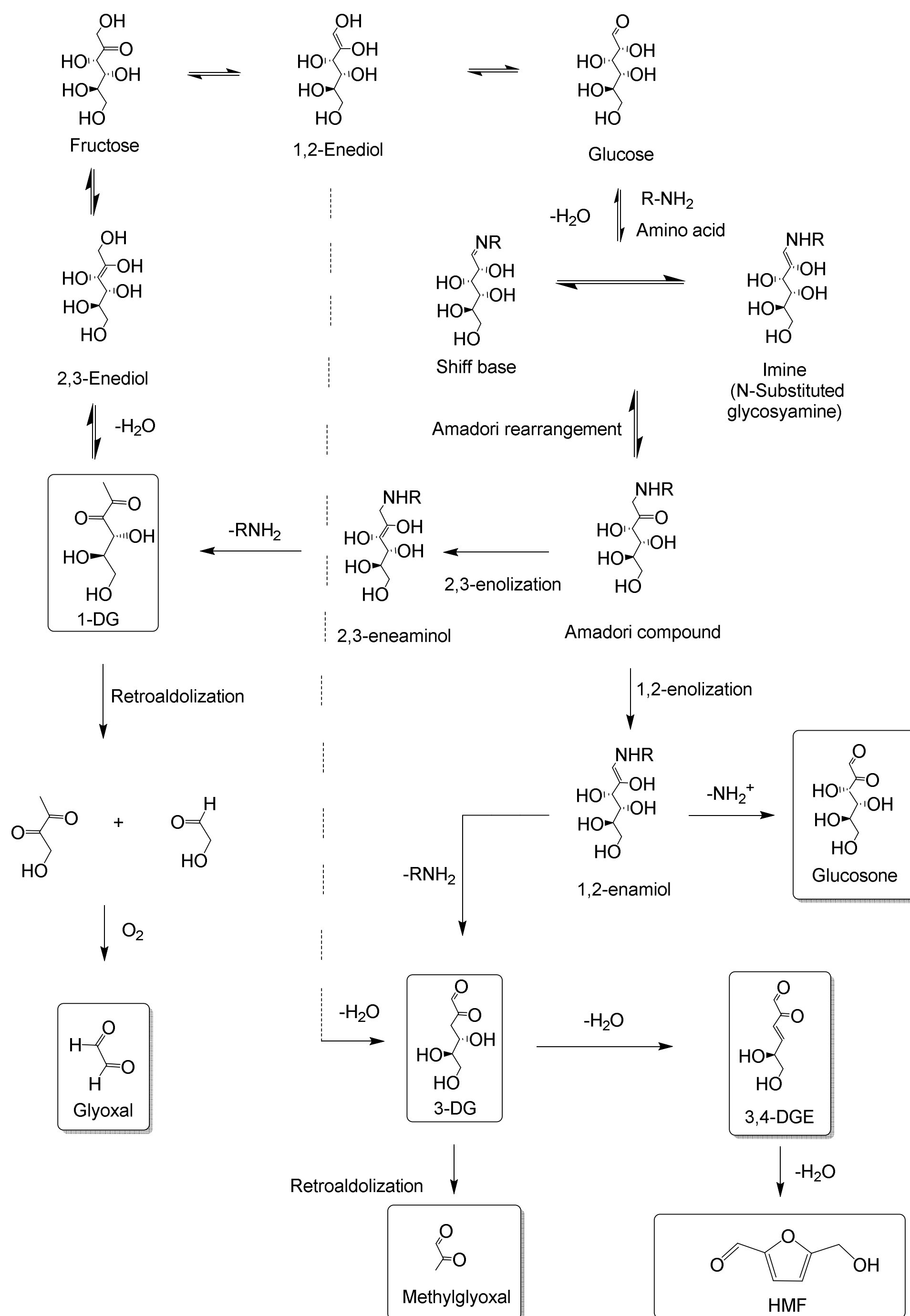


Figure 9. Formation of the most abundant carbohydrate-derived reactive dicarbonyls present in food (Amaya-Farfan & Rodriguez-Amaya, 2021; Hellwig *et al.*, 2018; Wong, 2018). Abbreviations: 3-DG, 3-deoxyglucosone; 3,4-DGE, 3,4-dideoxyglucosone-3-ene.

These carbonyls are also involved in the formation of other carbonyls. This occurs, for example, in the Strecker degradation of amino acids, which is an important source of pyrazines. Therefore, 1,2-dicarbonyls act like indirect aroma precursors (Scalone *et al.*, 2015). Formation of Strecker aldehydes will be discussed in section 1.6.3.

1.6.1.4. Factors influencing Maillard reaction

Some variables such as temperature, pH value, and water activity, play a major role in Maillard reaction yield. However, Maillard reaction is a complex process and these factors do not influence in the same way each food feature, including colour, flavour, or nutritional value.

A. Temperature

Temperature is the main factor in Maillard reaction. Maillard reaction rate increases with increasing temperature. The formation of secondary products of Maillard reaction such as Amadori compounds, melanoidins, and Strecker degradation products also increase as a function of heating temperature. The result of all these processes is an increasing rate of browning because more pigments are formed at higher temperatures (Eggleston *et al.*, 2018). However, Maillard reaction does not require high temperatures to observe some changes. Some signs of non-enzymatic browning are produced during prolonged storage (Arnoldi, 2004).

B. pH

Maillard reaction is favoured at pH $\geq$ 7, but, under acid conditions, the formation of some products can also be observed. Therefore, pH or the presence of a buffer has an important effect on the reaction. A change in the pH of the medium also produces changes in volatiles and coloured products. Thus, browning is faster in neutral foods and decreasing pH usually reduces the rate of colour formation (Arnoldi, 2004; Eggleston *et al.*, 2018).

C. Water activity (a_w)

Water content is also important. Maillard reaction proceeds much more easily at low or high moisture levels and it is generally accepted that an a_w around 0.6 is the less favourable moisture value to Maillard reaction yield (Arnoldi, 2004; Eggleston *et al.*, 2018).

D. System composition

The structure of the reactants, sugars and amino acids, also play a major role. The nature of the sugars determines their reactivity. Thus, simple reducing sugars react faster than oligosaccharides or complex sugars. Reactivity is also related to their conformational stability and to the amount of open-chain structure present in solution (Eggleston *et al.*, 2018).

Something similar occurs with the effect of the type of amino acid. Although free amino acids are not very abundant in most foods, they react easily with sugars. In the α-amino acid series, glycine is the most reactive. The structure of the side chain of the amino acid determines the rate of browning. Thus, longer and more complex substituent groups reduce the rate of browning (Arnoldi, 2004; Eggleston *et al.*, 2018).

1.6.1.5. Consequences of Maillard reaction

Consequences of Maillard reaction are both positive and negative. On one hand, Maillard reaction may influence positively flavour, aroma, colour, and texture of foods as a consequence of processing. On the other hand, Maillard reaction may also have negative consequences. Thus, it can develop undesirable colour and off-flavours, and produce carcinogenic or mutagenic compounds such as acrylamide (Muttucumaru *et al.*, 2014) or heterocyclic aromatic amines (Starowicz & Zieliński, 2019). These consequences are related to the formation of different compounds:

- The browning development is due to the production of melanoidins, which are undesirable in some food products. These polymers contain variable amounts of nitrogen and a wide variability of molecular weights and solubilities in water.
- The volatile products of the Maillard reaction are essential to the characteristic flavour and aroma of many food products. Desired aromas are usually produced during cooking, baking, roasting, or frying. For example, pyrazines contribute of the flavour of beef, cocoa, coffee, or peanuts, and furan derivatives give sweet and caramel aroma of bread, biscuits, jam, or honey. However, Maillard reaction may also generate off-flavours in foods during storage.
- Changes in food texture are also produced through protein cross-linking but this consequence has been lesser studied. Reactions of this type also play a role in vivo: Diabetic complications and cardiovascular and other diseases (Starowicz & Zieliński, 2019).
- Maillard reaction also produce compounds with antioxidant properties that have a protective effect (Nursten, 2005).

1.6.2. Reactive carbonyls produced from lipids

Lipids are also a major source of reactive carbonyls. These compounds are produced as a consequence of lipid oxidation. Although most food components are susceptible to oxidation, lipids are the most susceptible compounds to this deteriorative degradation. This is a consequence of the susceptibility of fatty acids to react with oxygen.

Lipid oxidation produces the reduction of the nutritional value of foods because essential fatty acids and vitamins are lost, and changes in sensory quality including colour, texture, and the production of rancid odours and flavours. In spite of this fact, the main problem of lipid oxidation is likely the formation of harmful compounds. The formation of these compounds have been related to the development in human beings of atherosclerosis, cancer, inflammation, and aging

processes, among others (Alfaia *et al.*, 2010; Broncano *et al.*, 2009; Pereira & Abreu, 2018).

1.6.2.1. Lipids

Lipids present in foodstuff come from the structural lipids of animal or plant food raw material. They are components of cell and organelle membranes, and are also present in depot fats (Domínguez-Avila & González-Aguilar, 2019; Sikorski & Sikorska-Wiśniewska, 2006).

Lipids are a broad group of structurally and functionally diverse compounds. They are hydrophobic in nature and, mainly, soluble in organic solvents. Fatty acids, phospholipids, sterols, and others, are some examples of lipids with these characteristics (Finley & DeMan, 2018). Lipids are classified in many ways. Thus, according to their physical properties at room temperature, they are classified into fats, when they are solid, and into oils, when they are liquid (Jambrak & Škevin, 2016). According to the origin, they are classified as animal (mammal depot fats, milk fats, and marine oils) or vegetable (seed oils, fruit coat fats, and kernel oils) fats (Finley & DeMan, 2018). According to their structures, they are classified into simple (e.g., acylglycerols) and complex lipids (e.g., glycerophospholipids) (Fahy *et al.*, 2011). Other important factor to take into in account is the health-promoting properties. These properties are related to the fatty acid composition and to the minor components (e.g., glycolipids, phospholipids, tocols, phytosterols, or phenolic compounds) (Jambrak & Škevin, 2016).

Oils and fats of plant and animal origins consist almost exclusively of triacylglycerols (TAG), with the chemical structure of glycerol esterified with three molecules of fatty acids. Fatty acids are, therefore, the major components of lipids. Their structure is a hydrocarbon chain of an even number of carbons and a carboxylic acid group at one end of the molecule. They are commonly classified into long chain (when the chain has more than 12 carbon atoms), medium chain

(between 8 and 12 carbon atoms), and short chain (between 4 and 6 carbon atoms) fatty acids. The most abundant fatty acids in most organisms have 16-18 carbon atoms. Based on the presence or not of double bonds, fatty acids can be unsaturated or saturated, respectively. In addition, according to the number of double bonds, fatty acids can be monounsaturated (one double bond) or polyunsaturated (several double bonds). Double bonds can occur in two forms *cis* and *trans*, but most fatty acids found in natural fats have *cis* configuration. In polyunsaturated fatty acids, also named PUFAs, double bonds are usually separated by one methylene group. Depending on the position of the first carbon-carbon double bond counted from the methyl end, fatty acids can be classified into families: ω3 (linolenic type), ω6 (linoleic type), ω7 (palmitoleic acid type), and ω9 (oleic acid type). Linolenic and linoleic acids are considered essential fatty acids for human beings and they cannot be produced within the human body. Chemical structures for some of these fatty acids are given in **Figure 10** (Finley & DeMan, 2018; Wong, 2018).

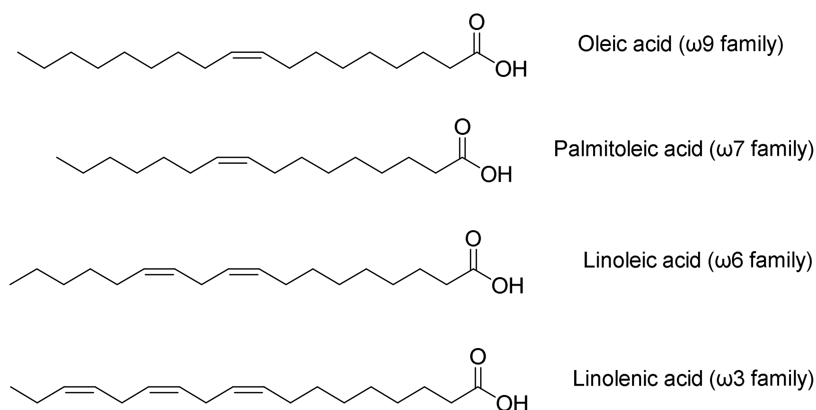


Figure 10. Structures of main fatty acids.

1.6.2.2. Lipid oxidation as a main pathway for the formation of lipid-derived reactive carbonyls

Lipid-derived reactive carbonyls include ketones and aldehydes with diverse structures (**Figure 11**) such as alkanals (Wu & Wang, 2019), 2-alkenals (Bastos

et al., 2017), 2,4-alkadienals (Beltrán *et al.*, 2011), 4-hydroxy-2-alkenals (Csallany *et al.*, 2015), 4-oxo-2-alkenals (Tullberg *et al.*, 2019), 4,5-epoxy-2-alkenals (Zamora *et al.*, 2017), and malondialdehyde (Bertolín *et al.*, 2019), among others (Elmore *et al.*, 2005). They are produced as a consequence of a cascade of reactions initiated by the reaction of fatty acyl chains with oxygen: The lipid oxidation.

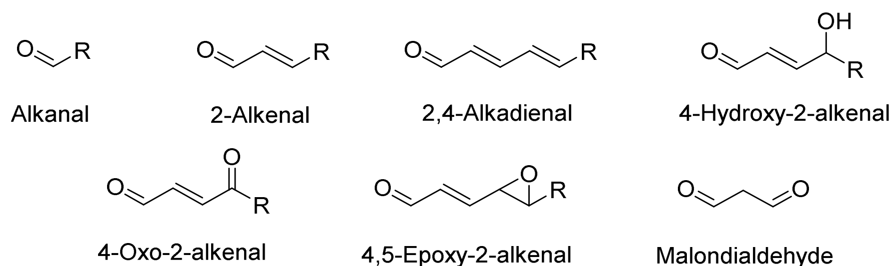


Figure 11. Main classes of lipid-derived reactive carbonyls found in food (Zamora & Hidalgo, 2020).

The reaction needs the presence of initiators. Among them, heat, free radicals, light, photosensitizing pigments, and metal ions play a major role. Depending of the initiators, lipid oxidation may be a photooxidation (induced by light), an enzyme reaction (initiated by lipoxygenase), or a free-radical reaction (autooxidation) (**Figure 12**). The three of them follow a similar scheme with slight differences (Finley & DeMan, 2018). The autooxidation is the most general pathway and will be described in this section.

The process of autoxidation is produced through sequential free radical chain reactions and, like any other free radical reaction, implies three stages: Initiation, propagation, and termination (Rodriguez-Amaya & Shahidi, 2021).

O_2

Initiation

RH → R• Propagation ROO•

RH

ROOH

Metal ion | hν or Δ

OH^- $OH^\bullet$

Monomeric compounds (epoxy-, hydroxy-, keto-derivatives) ← stabilization — RO• — polymerization → Polymeric compounds (RR, ROOR, ROR)

scission

Volatile compounds (aldehydes, hydrocarbons, alcohols, ketones)

Figure 12. Lipid oxidation pathway.

- Initiation

The initiation step involves the homolytic breakdown of the carbon-hydrogen bond at the α-position in relation to the double bond in the unsaturated acyl group (usually the bis-allylic carbon atom). This reaction produces the corresponding alkyl radicals ($R^\bullet$). The reaction is thermodynamically unfavourable and the presence of an initiator (light, heat, metal ions) is needed (Schaich *et al.*, 2013).

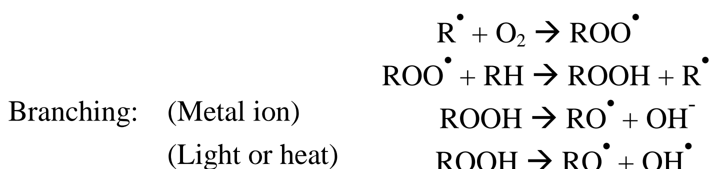
$$RH + Initiator \rightarrow R^\bullet$$

Hydrogens with lower bond dissociation energies are firstly removed. PUFAs are particularly susceptible to hydrogen abstraction due to the presence of single or multiple methylene-interrupted double bonds (McClements & Decker, 2018). Once produced, the resulting alkyl radical ($R^{\bullet}$) is stabilized by delocalization over the double bond(s) to form a conjugated diene (McClements & Decker, 2018).

- Propagation:

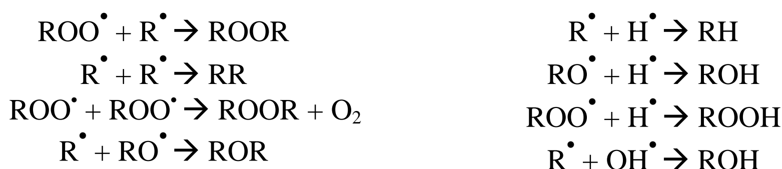
The propagation phase of oxidation occurs with the addition of molecular oxygen to the produced alkyl radical ($R^{\bullet}$) to form the corresponding peroxyl radical ($ROO^{\bullet}$). This radical is less reactive than the alkyl radical. Consequently, most free radicals are in the form of peroxyl radicals. At the same time, a hydrogen atom from another fatty acid molecule (RH) is transferred to the peroxyl radical to produce the corresponding hydroperoxide (ROOH) and an alkyl radical ($R^{\bullet}$). This new alkyl radical starts again the same process. This process continues indefinitely until no hydrogen source is available or the chain is interrupted.

Hydroperoxides are decomposed into alkoxyl radicals, peroxyl radicals, and hydroxyl radicals by metal ions, heat, and ultraviolet (UV) light. When hydroperoxide decompositions are catalysed by metal ions, the scissions are heterolytic, producing one radical and one ion. However, when hydroperoxide decompositions are catalysed by heat and UV light, homolytic scission is produced and two radicals, alkoxyl ($RO^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals, are produced. Both radicals react much more rapidly than $ROO^{\bullet}$. This second part of the propagation step is known as branching (McClements & Decker, 2018; Schaich *et al.*, 2013). A brief scheme reaction of the propagation step is:



- Termination:

As usual in all free radical reactions, free radical chain is broken when the collision of free radicals or the reactions between radicals with other non-radical compounds provoke the formation of stable molecules, thus making the free radicals disappear (McClements & Decker, 2018). Some of these reactions are:



These reactions are responsible for the formation of some secondary oxidation products. In addition, other secondary oxidation products are produced as a consequence of the breakage of free radicals. This will be discussed in the next subsection.

1.6.2.3. <u>Formation of lipid oxidation products</u>

As indicated above, there are many routes and also many starting materials. Therefore, the number of produced compounds is large. They are named as lipid oxidation products (LOPs) and their formation occurs sequentially. For this reason, some of them are called primary LOPs and other are named secondary LOPs. In addition, the existence of tertiary LOPs is also considered by some authors (Lu *et al.*, 2014).

- Primary LOPs.

Lipid peroxidation generates hydroperoxides in the propagation and termination steps, which are considered as the primary LOPs. Despite these compounds have not a role on food flavour, they are unstable and they have deleterious effects on health. For most foods, the main unsaturated fatty acids present in them and susceptible to oxidation are: Linoleic (LH) and linolenic acids (LnH). Their autooxidation produces the corresponding hydroperoxides: 9-

LOOH and 13-LOOH from linoleic acid and 9-LnOOH and 13-LnOOH from linolenic acid (Rodriguez-Amaya & Shahidi, 2021; Wong, 2018). **Figure 13** shows the hydroperoxides formed as a consequence of linoleic acid oxidation.

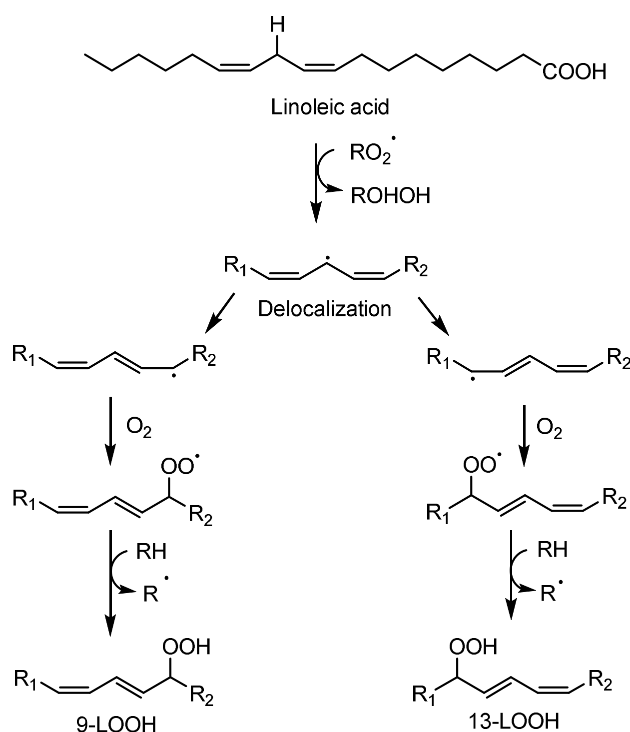


Figure 13. Lipid oxidation products (LOPs) formation from linoleic acid R_1 = C_5H_{11} and R_2 = $C_8H_{15}O_2$.

- Secondary LOPs.

Secondary LOPs are formed from hydroperoxides, which can be decomposed in the presence of metal ions or at high temperatures. Primary LOPs are non-volatile compounds and they are odourless and tasteless but they are easily degraded into a high number of volatile and non-volatile compounds, such as carbonyls (e.g. ketones and aldehydes), alcohols, hydrocarbons (e.g. alkanes, alkenes), and furans, that play a major role in the flavour of food products (Bastos *et al.*, 2017; McClements & Decker, 2018; Yin *et al.*, 2020) (**Figure 14**).

In fact, their formation has been related to deterioration of quality and production of rancidity, harmful substances, and discoloration (Domínguez *et al.*, 2019).

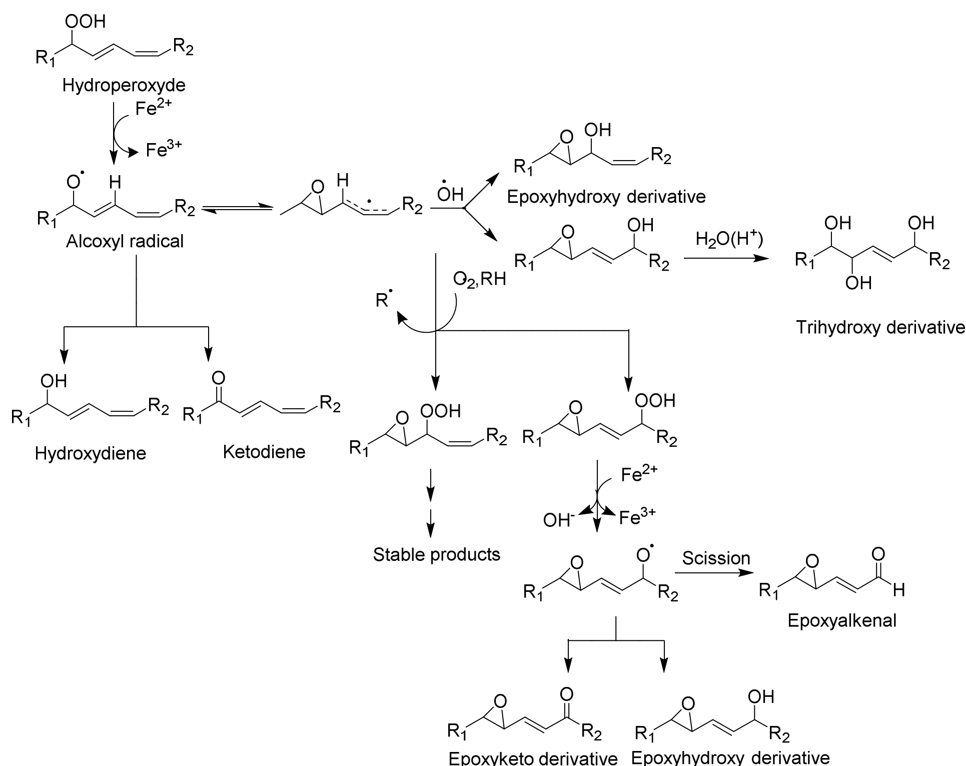


Figure 14. Degradation of linoleic acid hydroperoxides to hydroxy-, epoxy- and oxo-fatty acids. Only segments of the structures are presented. $R_1 = C_5H_{11}$ and $R_2 = C_8H_{15}O_2$ (Belitz *et al.*, 2009).

Theses secondary LOPs are classified into three main groups (Berdeaux *et al.*, 2012):

- Low molecular weight volatile compounds formed through hydroperoxide decomposition. The main route to produce these LOPs is the homolytic β-scission of the alkoxyl radicals derived from allylic hydroperoxides. The alkoxyl radical undergoes C–C bond cleavage on either side of the alkoxyl group. The produced radical reacts then with either other radicals

or neutral molecules and either gains or losses one atom of hydrogen to form hydroxy- or keto-acids. **Figure 15** shows the general scheme of the alkoxyl-radical β-scission during unsaturated fatty acid oxidation.

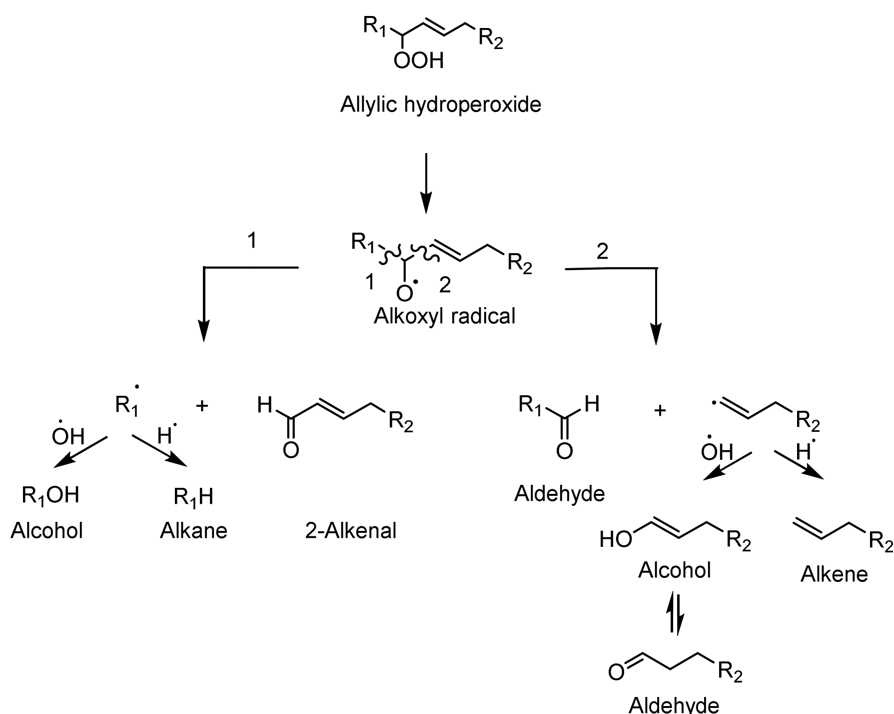


Figure 15. Some compounds produced by β-scission of alkoxyl radicals.

- Oxidized monomers of triglycerides are compounds with molecular weights similar to those of the non-oxidized triacylglycerols, but with an oxygenated function. This function is produced by transformation of the hydroperoxy group. The produced functional groups are epoxy, hydroxyl, or keto groups (**Figure 14**) (Belitz *et al.*, 2009; Wong, 2018).
- Dimers and oligomers of triacylglycerols formed by triacylglycerol radical polymerization reactions (Berdeaux *et al.*, 2012).

- Tertiary LOPs.

Secondary lipid oxidation products can suffer further oxidations and new compounds with two oxygenated functions are produced. These compounds are analogous to the above described 1,2-dicarbonyl derivatives (section 1.6.1.3) and diverse combinations of functional groups have been described: Epoxyketo, epoxyhydroxy, and ketohydroxy (Lu *et al.*, 2014). They can have the same chain length of the starting fatty acid or have been broken and being short-chain carbonyl compounds. Thus, for example, the decomposition of polyunsaturated fatty acids, produce 4,5(*E*)-epoxy-2(*E*)-heptenal or 4-oxo-2-hexenal when starting from ω3 fatty acyl chains and 4,5(*E*)-epoxy-2(*E*)-decenal or 4-oxo-2-nonenal when starting from ω6 fatty acyl chains (**Figure 16**). These products are common in food systems and play a major role in the colour and flavour changes produced in foods as a consequence of lipid oxidation (Hidalgo & Zamora, 2004).

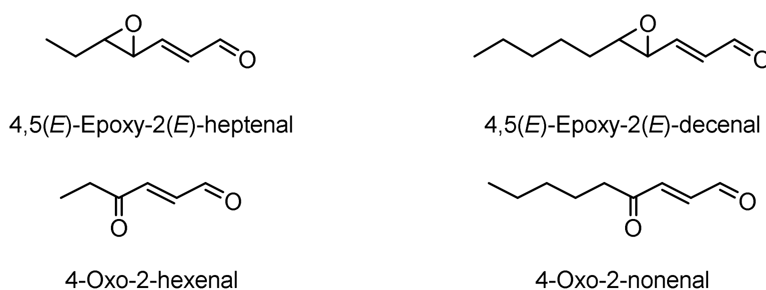


Figure 16. Structures of some tertiary lipid oxidation products from ω3 (left) or ω6 (right) polyunsaturated fatty acids.

1.6.2.4. <u>Factors influencing lipid oxidation rates</u>

Lipid oxidation depends on many factors, including lipid unsaturation degree, temperature, presence of prooxidants, antioxidants, amount of oxygen, water activity, distribution of lipids in the food, chelators and sequestering agents, light, surface area, and pH (Finley & DeMan, 2018; Wong, 2018). Some of these factors are discussed below:

A. Temperature

Lipid oxidation increases exponentially as a function of temperature (Frankel, 2014). In addition, increasing temperature produces the decomposition of hydroperoxides, which promotes the propagation phase.

Temperature is strongly related to the formation and decomposition rates of primary LOPs. At 130–140ºC, decomposition prevails over formation rates. So, the most important products at elevated temperatures are secondary LPOs due to the higher stability of some of them at such temperatures (Dobarganes *et al.*, 2016; Dobarganes & Márquez-Ruiz, 2007; Velasco & Dobarganes, 2002). Besides the rates, temperature also determines the distribution of LOPs.

Formation rates of different groups of compounds when the food is processed at high temperature also depend on how that temperature has been applied. For example, roasting applied for a longer time at a lower temperature produces a higher increase in secondary lipid oxidation products than heating at a higher temperature for a shorter time (Broncano *et al.*, 2009).

On the other hand, although freezing slows down lipid oxidation, it does not stop the process. Extracellular ice crystal formation by temperature fluctuations promotes oxidation as a result of an increasing cell disruption, which release prooxidant compounds (Erickson, 2002). Moreover, oxygen solubility in liquids decreases when temperature increases and changes in partitioning of antioxidants between phases are also produced (Gordon, 2004).

B. Antioxidants

Antioxidants are compounds that protect lipids from oxidation. They delay or slow the rate of oxidation by inhibiting the action of catalysts or the formation of free radicals (Pereira & Abreu, 2018). Antioxidants naturally present in foods include tocopherols, ascorbic acid, carotenoids, peptides, and enzymes. In addition, they can also be added to the food system (Carocho & Ferreira, 2013).

The effects of antioxidants on the oxidation of foods are dependent on their concentration, polarity, the medium, and/or the presence of other antioxidants (Cuvelier *et al.*, 2000; Elias & Decker, 2017; Frankel *et al.*, 1996; Samotyja & Małecka, 2006). To be considered an antioxidant, the compound must delay the oxidation process at a low concentration in relation to the concentration of the oxidizable compound. In addition, at high concentrations, many antioxidants behave as prooxidants (Halliwell & Gutteridge, 2015).

To be employed as food additive, an antioxidant needs (Schuler, 1990):

1. To be inexpensive, nontoxic, and effective at low concentrations.
2. To have a high stability and capability of surviving to food processing.
3. To have no odour, taste, or colour.
4. To be easy to incorporate and to have a good solubility in the product.

C. Amount of oxygen present

Oxygen is a key component in lipid oxidation. Their concentration is essential to control the oxidative process. When unlimited oxygen is available, the rate of oxidation does not depend on it. However, at low concentrations of oxygen, the oxidation rate is approximately proportional to oxygen concentration. For this reason, a method to inhibit lipid oxidation is the reduction of oxygen concentration.

The addition of oxygen to the alkyl radical is a diffusion-limited reaction, so it has been suggested that to effectively inhibit lipid oxidation, most of the oxygen must be removed from the system. The effect of oxygen concentration on oxidation rate is also influenced by other factors, such as temperature and surface area. In addition, oxygen solubility is higher in oil than in water. Therefore, removal of oxygen to stop lipid oxidation can be difficult, unless vacuum

conditions are used or oxygen is completely replaced by an inert gas (e.g. nitrogen) (McClements & Decker, 2018).

D. Water activity (a_w)

Water activity also has a significant impact on lipid oxidation in low moisture foods. Lipid oxidation proceeds very rapidly in foods with high content of water. Then, oxidation rates usually decrease when water is removed from the food system. This is likely because of a decrease in the mobility of reactants such as transition metals and oxygen. Nevertheless, in some foods, continued removal of water results in an acceleration of lipid oxidation. This acceleration at low water activity ($a_w \leq 0.3$) has been related to the loss of a protective water solvation layer surrounding lipid hydroperoxides (McClements & Decker, 2018).

1.6.2.5. Consequences of lipid oxidation

Lipid oxidation is one of the major causes of food deterioration. However, in some cases, lipid oxidation is used to increase product quality, for example the enzymatic production of fresh-fish aromas (Erickson, 2002). Consequences of lipid oxidation can be classified into two groups according to the involved mechanism. Thus, changes in food properties can be a direct consequence of changes produced in lipids: Production of off-odors and flavours, degradation of membranes, or alteration of lipid structures. In addition, changes can also be due to interactions of lipid oxidation intermediates or products with other molecules, such as reactions of lipid radicals, hydroperoxides, epoxides, or aldehydes with amino acids, proteins, or phenolics, among other compounds (Schaich *et al.*, 2013).

Lipid physical structures in foods can also be modified or deteriorated by lipid oxidation. When lipids are oxidized, the hydrophobic associations among acyl chains are disrupted. In addition, polar compounds are accumulated, cross-linking among fatty acyl chains are produced, and acyl chains structural

organization is lost. In foods, these changes are shown as losses of turgor and degradation of the texture.

When, lipid oxidation products interact with other food components, food quality can also be degraded. For example, although rancidity is usually only attributed to lipids, it might be the result of reactions of lipid oxidation intermediate radicals and LOPs with other molecules. Thus, many off-flavours attributed to lipids, e.g. warmed-over flavour in meats and some stale flavours, arise from co-oxidations. One important factor that complicates sorting out co-oxidations is that nearly all lipid oxidation intermediates and products, especially radicals, hydroperoxides, aldehydes, and epoxides react with proteins, starches, pigments, DNA, and other molecules (Schaich *et al.*, 2013).

1.6.3. Reactive carbonyls produced from amino acids

Analogously to carbohydrates and lipids, amino acids are also a source of reactive carbonyls.

1.6.3.1. Amino acids

The chemical structure of amino acids is shown in **Figure 17**. As can be observed, amino acids present in proteins are characterized by one amino and one carboxylic group bonded to the carbon at the α-position in the relation to the carboxylic group.

Food proteins are composed by combination of 20 amino acids. They differ in the side chain. The simplest, glycine, does not have a side chain. Other amino acids are more complex and this side chain can be either an aliphatic, aromatic or heterocyclic group. As a result, each amino acid has unique characteristics including net charge, solubility, chemical reactivity, and hydrogen bonding potential.

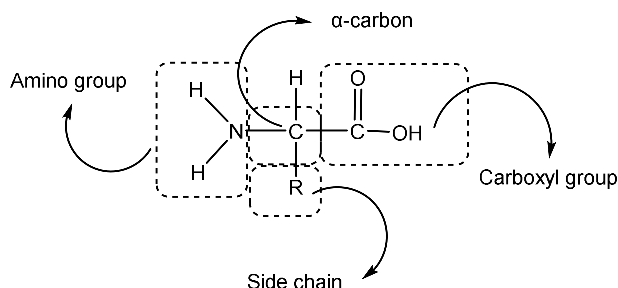


Figure 17. General structure of amino acids.

Some amino acids cannot be synthesised by human beings. They have to be taken with the diet. They are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. In addition, other amino acids are considered conditionally essential because their biosyntheses can be limited under certain conditions (arginine, cysteine, glycine, glutamine, proline, and tyrosine). Finally, not-essential amino acids are alanine, aspartic acid, glutamic acid, serine, and asparagine (Appell *et al.*, 2018).

These amino acids play an important role in food quality. Thus, they participate in the production of important taste and flavour volatiles compounds (Strecker aldehydes, pyrazines, pyridines, pyrroles, and oxazoles). On the other hand, amino acids can also have negative consequences in food quality. Thus, for example, amino acid decarboxylation produces amino compounds, some of which have harmful properties (Hidalgo *et al.*, 2016).

A. Strecker degradation of amino acids

One of the most important pathways for flavour formation through carbonyl-amine reactions is the Strecker degradation of amino acids. This reaction produces the conversion of amino acids into Strecker aldehydes, α-keto acids, and amines, which are important flavours in foods (Hidalgo & Zamora, 2004; Rizzi, 2008; Yaylayan, 2003). These conversions are produced by reactions between the amino group of amino acids and carbohydrate-derived 1,2-

dicarbonyl compounds, lipid-derived reactive carbonyls (Hidalgo *et al.*, 2016; Hidalgo & Zamora, 2004), and quinones produced by polyphenol oxidation (Hidalgo, *et al.*, 2013b). LOPs involved in amino acid degradation usually have two conjugate oxygenated functions (an epoxy or hydroxyl group and a carbonyl group) (Hidalgo & Zamora, 2004, 2016; Zamora & Hidalgo, 2011). Alternatively, amino acid degradations are also produced by the thermal decomposition of amino acids (Hidalgo, *et al.*, 2013a).

- Conversion of α-amino acids into Strecker aldehydes.

Strecker degradation of amino acids involves the reaction between an amino acid and a reactive carbonyl compound. As an example, **Figure 18** shows the Strecker degradation of the methionine.

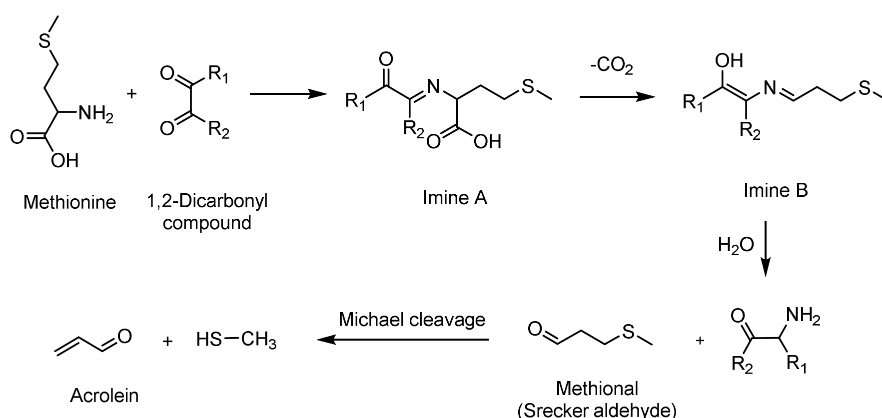


Figure 18. Strecker degradation of methionine and later conversion of methional into acrolein (Zirak *et al.*, 2019).

As shown in the figure, the pathway has several steps. It starts with the reaction between methionine and the 1,2-dicarbonyl compound. The result of this reaction is the formation of an imine (imine A), which suffers a decarboxylation and produces a new imine (imine B). The hydrolysis of this last imine is the origin of methional (the Strecker aldehyde of methionine). This aldehyde can also suffer the elimination of methanethiol and be converted into acrolein. This

last aldehyde can also have other origins. Thus, acrolein can also be formed from threonine during heat treatment of foods via Strecker degradation. In this case, 2-hydroxypropanal is the Strecker aldehyde, which is converted into acrolein by loss of one water molecule (Guth *et al.*, 2013; Stevens & Maier, 2008; Zirak *et al.*, 2019).

- Conversion of α-amino acids into α-keto acids.

A similar pathway to that described for the formation of Strecker aldehydes is the origin of α-keto acids (**Figure 19**). In this mechanism, the decarboxylation of the amino acid is not produced. In fact, the electronic rearrangement of the initial imine A produces an imine B without the loss of carbon dioxide. After the formation of imine B, α-keto acids are produced by hydrolysis. However, α-keto acids are not thermally stable compounds and they can be degraded into Strecker aldehydes or shorter aldehydes (Hidalgo & Zamora, 2016; Zamora & Hidalgo, 2011).

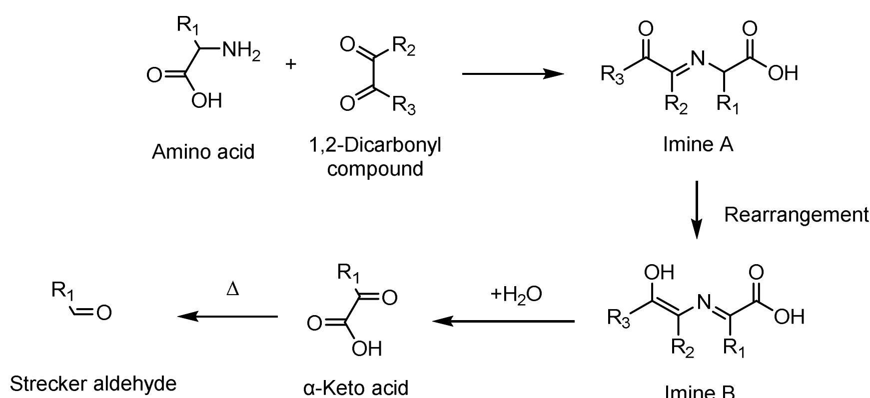


Figure 19. Conversion of amino acids into α-keto acids.

B. Formation of reactive carbonyls by thermal degradation of amino acids

In addition to the reaction of amino acids with lipids, carbohydrates or phenolics, amino acids can also be the origin of carbonyl compounds by thermal decomposition. Thus, as indicated **Figure 20**, the thermal degradation of alanine

or serine produces carbonyl compounds, such as acetaldehyde and/or formaldehyde.

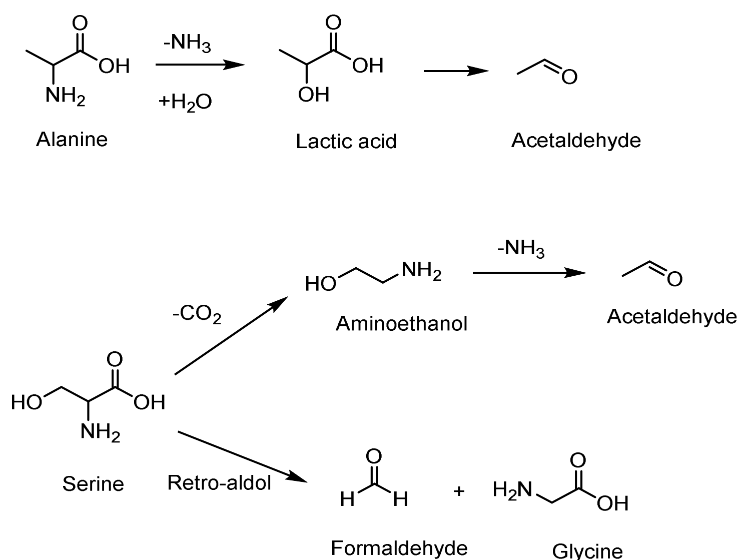


Figure 20. Thermal degradation of amino acids (Yaylayan *et al.*, 2000).

1.6.4. Reactive carbonyls produced from phenolic compounds

1.6.4.1. Phenolic compounds

Phenols are a large group of compounds characterized for having at least one aromatic ring with one or more hydroxyl groups attached. They are associated with the nutritional and sensory quality of fresh and processed plant foods, and they are found in many herbs, fruits, vegetables, grains and cereals, green and black teas, coffee beans, propolis, and red and white wines (Ho, 1992).

Phenolics can be classified into three groups according to the number and arrangement of their phenolic subunits. These three groups are simple phenols or phenolic acids, hydroxycinnamic acid derivatives, and polyphenols, which possess at least two phenol subunits. This last group includes flavonoids and stilbenes, among others.

Simple phenols include monohydroxylic derivates such as *p*-cresol, *o-, m-,* and *p*-diphenols such as catechol, resorcinol, and hydroquinone, respectively, and triphenols such as gallic acid. Hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic, and sinapic acids. Finally, flavonoids are the largest group of polyphenols in food, and there are an estimated number of 4,000 compounds of this kind known. They possess two aromatic rings, each containing at least one hydroxyl group, which are connected through a three-carbon "bridge" (Shahidi *et al.*, 2019).

1.6.4.2. Quinones as reactive carbonyls and as a source of them

Analogously to carbohydrates and lipid oxidation products, polyphenols are also able to produce the Strecker degradation of amino acids (Rizzi, 2008). In addition, they participate as antioxidants during the lipid oxidation process and contribute to the flavours of foods (Delgado *et al.*, 2015; Ho, 1992; Shahidi & Ambigaipalan, 2015).

Although there are a high number of polyphenols, only a few of them are able to produce the Strecker degradation of amino acids. The structural characteristics required for this reaction are the presence of, at least, two hydroxyl groups in *ortho-* or *para-* positions. However, the presence in the same molecule of other aromatic ring with two hydroxyl groups in *meta-* position either cancelled or limited the ability of these complex phenols to degrade amino acids (Delgado *et al.*, 2015).

The reason for the required structural characteristics is the need that these compounds are converted into quinones. Quinones are 1,2-dicarbonyl compounds and react with amino acids analogously to other 1,2-dicarbonyl compounds (**Figure 21**). Thus, they form the corresponding imine in the initial step, which after decarboxylation and hydrolysis produce the Strecker aldehyde at the same time that the phenolic compound is converted into a hydroxyphenylamine.

Quinones can also be produced by fermentative processes of foods as observed in tea, coffee, and cacao (Rizzi, 2008).

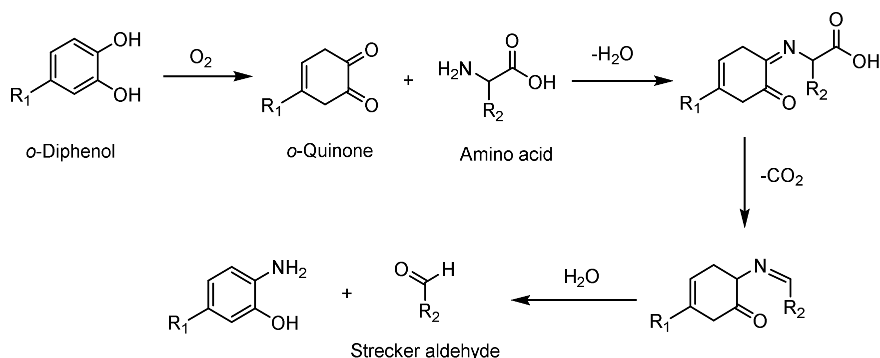


Figure 21. Contribution of *o*-quinones to degradation of amino acids (Schieber, 2018).

1.7. Disappearance of reactive carbonyls in foods: Food carbonylome fate

As discussed in the previous section, reactive carbonyls have different origins and are formed by different pathways. However, many of these compounds are highly reactive and they take part in different reactions, which produces their rapid clearance. Some of these reactions will be discussed in this section.

1.7.1. Carbonyl–amine reactions

An important fate of reactive carbonyls is carbonyl-amine reactions. These reactions are produced with the different reactive carbonyls described in the previous section. However, these reactions have been traditionally considered different depending on the origin of the carbonyl compounds.

Thus, carbonyl-amine reactions produced as a consequence of carbohydrate/amino acids reactions have been named Maillard reaction. In this reaction, when carbohydrate-derived reactive carbonyls react with amines, amino acids, aminophospholipids, and proteins, advanced Maillard reaction products are

produced. These products are also known as advanced glycation end-products (AGEs) (Uribarri *et al.*, 2010).

The structure of the produced AGEs (**Figure 22**) depends on the nature of the amino moiety. Thus, when the amine group is attached to a protein, the reaction can result in cross-linking or unchangeable modifications of the protein, which may lead to functional alteration. For example, the reaction of a pentose with lysine (Lys) and arginine (Arg) residues of proteins produces a cross-linker, named pentosidine. Others examples of cross-linkers are those resulting from the reaction between two lysine side-chains and two molecules of 1,2-dicarbonyl compounds, such as glyoxal (GO), methylglyoxal (MGO), or 3-deoxyglucosone (3-DG). The formed AGEs are glyoxal-derived lysine dimer (GOLD), methylglyoxal-derived lysine dimer (MOLD), and 3-deoxyglucosone-derived lysine dimer (DOLD), respectively. The crosslinking between arginine and lysine results in GODIC (imidazolium cross-link derived from glyoxal and lysine-arginine), MODIC (imidazolium cross-link derived from methylglyoxal and lysine-arginine), and DODIC (imidazolium cross-link derived from 3-deoxyglucosone and lysine-arginine) (Akıllıoğlu & Gökmen, 2019).

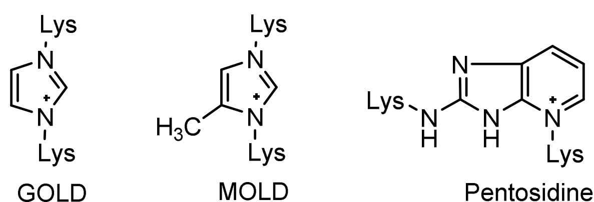


Figure 22. Examples of advanced glycation end-products (AGEs).

Other AGEs do not produce cross-linking. The most common non-cross-linking AGEs are pyrraline and N^{ε}-carboxymethyllysine (CML), which is used as a marker of the AGEs formed in food. CML can be formed through different pathways. Thus, CML is produced by condensation of glucose with the ε-amino group of lysine, by reaction of GO directly with the ε-amino group in lysine, and through the oxidation of fructoselysine (Poulsen *et al.*, 2013; Yu *et al.*, 2017).

Analogously to GO, methylglyoxal forms N^{ε}-carboxyethyllysine (CEL). CEL and CML are the better-studied AGEs and together with pentoside and pyrraline are usually used as indicators of the nutritional quality of foodstuffs. The high consumption of sugars, processed foods, and of fats or proteins have resulted in an increased exposure to AGEs.

As discussed above, carbohydrates are not the only source of reactive carbonyls in foods. Lipid oxidation is also a major source of reactive carbonyls, which compete with carbohydrate-derived reactive carbonyls for amino compounds. Therefore, a major cause for lipid-derived reactive carbonyls disappearance is also the production of carbonyl-amine reactions (Hidalgo & Zamora, 2000).

Lipid-derived reactive carbonyls react with amines, amino acids, peptides, proteins, and aminophospholipids, and produce advanced lipoxidation end products (ALEs) (Hidalgo & Zamora, 2017). Analogously to AGEs, ALEs can be classified into two groups: Low and high molecular weight products. Low molecular weight ALEs include ε-*N*-pyrrolylnorleucine. This compound has been found in many fresh food products and its content is directly correlated with lipid and iron contents (Zamora *et al.*, 1999). Other low molecular weight ALEs are heterocyclic derivatives, such as pyrroles and pyridines. Some of these low molecular weight ALEs are stable. However, others are unstable and polymerize spontaneously to produce high molecular weight ALEs, which are responsible for the browning developed by fat-rich foods upon processing and storage (Zamora & Hidalgo, 2005).

Foods with the highest levels of AGEs/ALEs are red meats and poultry. In addition, higher-fat and aged cheeses contain more AGEs/ALEs than lower-fat cheeses. It is remarkable the large amounts of AGEs/ALEs in these foods in spite of being uncooked foods. This is likely due to pasteurization and/or holding times at room temperatures (e.g., as in curing or aging processes). High-fat spreads also have high contents of AGEs/ALEs. However, grains, legumes,

breads, vegetables, fruits, and milk have lower contents of AGEs/ALEs (Uribarri *et al.*, 2010).

Analogously to reactive carbonyls from carbohydrates and lipids, quinones also react with amino acids and proteins through carbonyl-amine reactions.

Polyphenols are oxidized by polyphenol oxidases (PPOs). Polyphenol oxidases catalyse the oxidation of the colourless phenols to coloured quinones at the expense of O_2. These quinonic compounds are associated reversibly or irreversibly with amino acids.

Quinones react with the free nucleophilic functional groups of proteins or amino acids such as sulfhydryl, amine, amide, indole and imidazole substituents (**Figure 23**).

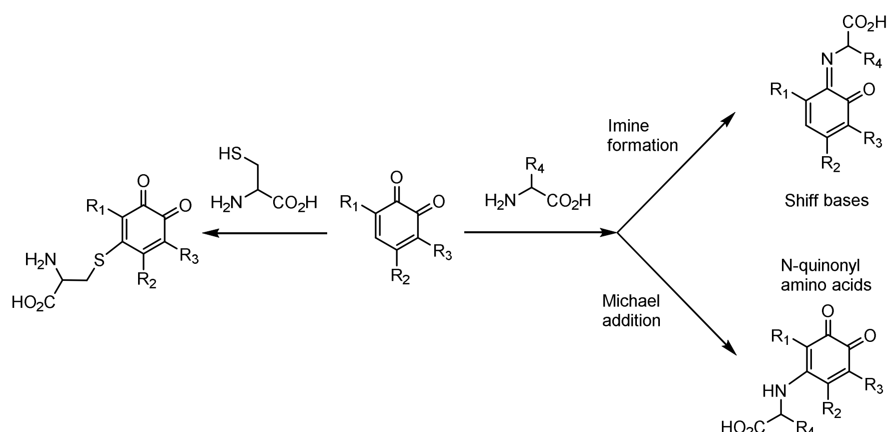


Figure 23. **N**on-enzymatic reactions between oxidised polyphenols and amino acids (adapted from Yaylayan, 2003).

It results in imine formation in 1,4-Michael addition via nitrogen or sulphur, and in Strecker degradation forming aldehydes. Thus, primary products are Schiff bases, *N*-quinonyl derivatives, *S*-quinonyl derivatives, and simple aldehydes evolving from the Strecker degradation, which have been described in subsection 1.5.4.2 (Yaylayan, 2003).

The formation and activity of quinone-amino acids conjugates influence the colour, taste, and aroma of foods. For example, chlorogenic acid (**Figure 24**) is the major polyphenol in foods derived from plants and is a good substrate for polyphenol oxidase. Chlorogenic acid quinone (**Figure 24**), which is the oxidative product of chlorogenic acid by polyphenol oxidase is an important intermediate compound in enzymic browning. In addition to the reaction of chlorogenic acid quinone with amino acids, H_2O_2 might also play an important role in the formation of the brown colour by enzymic browning (Bittner, 2006).

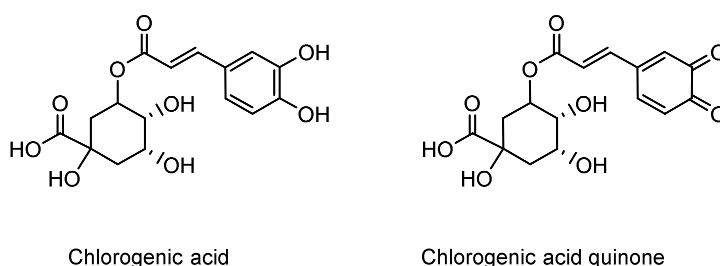


Figure 24. Structure of chlorogenic acid and chlorogenic acid quinone (Bittner, 2006).

Quinones can also be involved in reactions with proteins. They can react as electrophilic compounds, or undergo redox cycling and cause oxide reductive stress. Thus, quinones can react as Michael acceptors to alkylate proteins. In addition, quinones also react with sulphur nucleophiles, such as glutathione (GSH) or cysteine residues on proteins, or with nucleophilic amino groups of proteins (Bolton & Dunlap, 2017; Klopčič & Dolenc, 2019).

p-Quinones react via 1,4-reductive addition reactions regenerating the hydroquinone with covalent attachment to the cysteine residue. Unsubstituted *o*-quinones generally undergo 1,6-reductive addition reactions with thiol nucleophiles due to extended conjugation, although 1,4-reductive addition is often observed as a minor product (**Figure 25**). Reaction with nitrogen nucleophiles such as lysine, histidine, *N*-terminal amino acids, and purine and pyrimidine bases on DNA are much slower compared to that of sulphur

nucleophilic additions, although they occur to some extent. Generally, reductive 1,4-Michael additions predominate over Schiff base formation (Bolton & Dunlap, 2017).

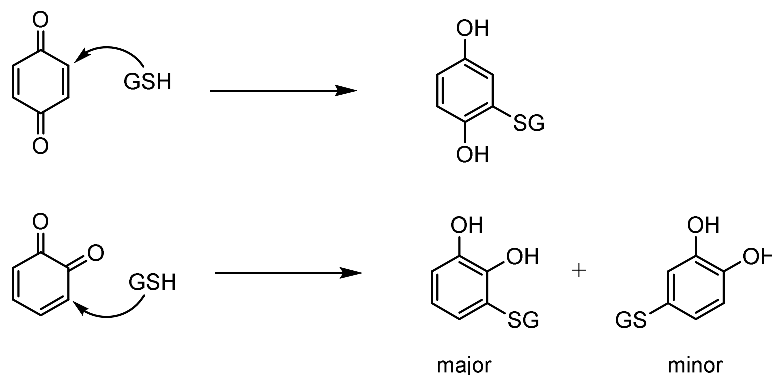


Figure 25. Michael additions of glutathione (GSH) to *p*-quinone and *o*-quinone (Bolton & Dunlap, 2017).

1.7.2. Carbonyl-phenol reactions

Analogously to amino compounds, phenolic compounds are also able to trap reactive carbonyls. The carbonyl scavenging ability of phenolic compounds has been long studied. Thus, studies in model systems observed the influence of phenolic compounds on the formation of food flavours during Maillard reaction (Totlani & Peterson, 2005). Moreover, recent studies have shown that phenolic compounds can also influence the formation of advanced glycation end-products (AGEs) and of advanced lipoxidation-end products (ALEs) by trapping or scavenging reactive carbonyls (Hidalgo, *et al.*, 2017b; Hidalgo, *et al.*, 2018b).

Most studies have been carried out with glyoxal (GO) and methylglyoxal (MGO). When these compounds are in the presence of phenolic compounds, they are trapped and the formation of AGEs and ALEs is inhibited (Zhu *et al.*, 2020). The trapping ability of phenolic compounds is a consequence of their structural characteristics (Hidalgo, *et al.*, 2017a; Zamora *et al.*, 2017).

Trapping sites of MGO molecules are known in the case of flavonoids (Lund & Ray, 2017). The requirements for the trapping ability of phenolic compounds are briefly summarized (**Figure 26**):

- The positions with the highest electronic densities are the carbons at the α-position to the carbons with the hydroxyl group in the phenolic ring. *Meta* configuration of the phenol is more reactive than *ortho* or *para* configurations.
- The hydroxyl group at C-5 on the A-ring enhances trapping efficiency.
- The double bond between C-2 and C-3 on the C ring could facilitate the trapping efficiency.
- The number of hydroxyl groups on the B-ring does not significantly influence the trapping efficiency.

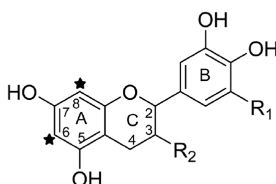
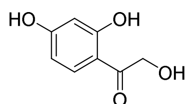


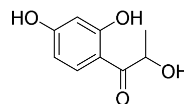
Figure 26. Structure of flavan-3-ols (Lund & Ray, 2017).

In addition to the ability of phenolic compounds to scavenge carbohydrate-derived reactive carbonyls, they can also form carbonyl-phenol adducts with lipid-derived reactive carbonyls, such as alkanals (Hidalgo, *et al.*, 2017a), 2-alkenals (Hidalgo & Zamora, 2014), 2,4-alkadienals (Hidalgo & Zamora, 2018), 4-hydroxy-2-alkenals (Hidalgo & Zamora, 2019), 4-oxo-2-alkenals (Hidalgo, *et al.*, 2018a), and 4,5-epoxy-2-alkenals (Zamora *et al.*, 2017). These carbonyl-trapping reactions have been shown to occur in foods under common cooking conditions (Zamora *et al.*, 2016). The structures of the identified carbonyl-phenol adducts produced with the different reactive carbonyls are shown in **Figure 27**:

Carbohydrate-derived carbonyl-phenol adducts

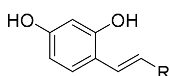


1-(2,4-Dihydroxyphenyl)-2-hydroxyethan-1-one

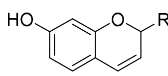


1-(2,4-Dihydroxyphenyl)-2-hydroxypropan-1-one

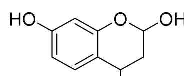
Lipid-derived carbonyl-phenol adducts



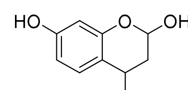
4-(Alk-1-en-1-yl)benzene-1,3-diol



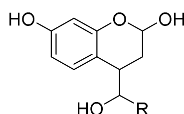
2-Alkyl-2*H*-chromen-7-ol



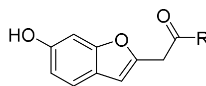
4-Alkylchromane-2,7-diol



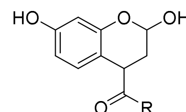
4-(Alk-1-en-1-yl) chromane-2,7-diol



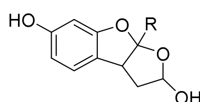
4-(1-Hydroxyalkyl)chromane-2,7-diol



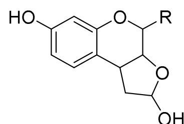
1-(6-Hydroxybenzofuran-2-yl)-alkan-2-one



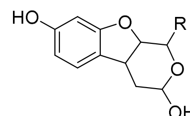
1-(2,7-Dihydroxychroman-4-yl)ethan-1-one



8a-Alkyl-2,3,3a,8a-tetrahydrofuro[2,3-*b*]benzofuran-2,6-diol



4-Alkyl-1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diol



1-Methyl-3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diol

Figure 27. Structures of the carbonyl-phenol adducts (Zamora & Hidalgo, 2020).

1.7.3. Cyclizations of reactive carbonyls in the presence of amino compounds

After reacting with amine groups, reactive carbonyls are modified and they usually evolve into heterocyclic derivatives. This happens, for example, in the Strecker degradation of amino acids (**Figure 28**).

As discussed previously, Strecker degradation (I) produces Strecker aldehydes and α-aminoketones. The formed α-aminoketones are unstable and dimerize spontaneously to produce pyrazines (II). As observed in the **Figure 28,** the

produced Strecker aldehyde can also be involved in these reactions (Scalone *et al.*, 2015, 2019; Zhao *et al.*, 2020).

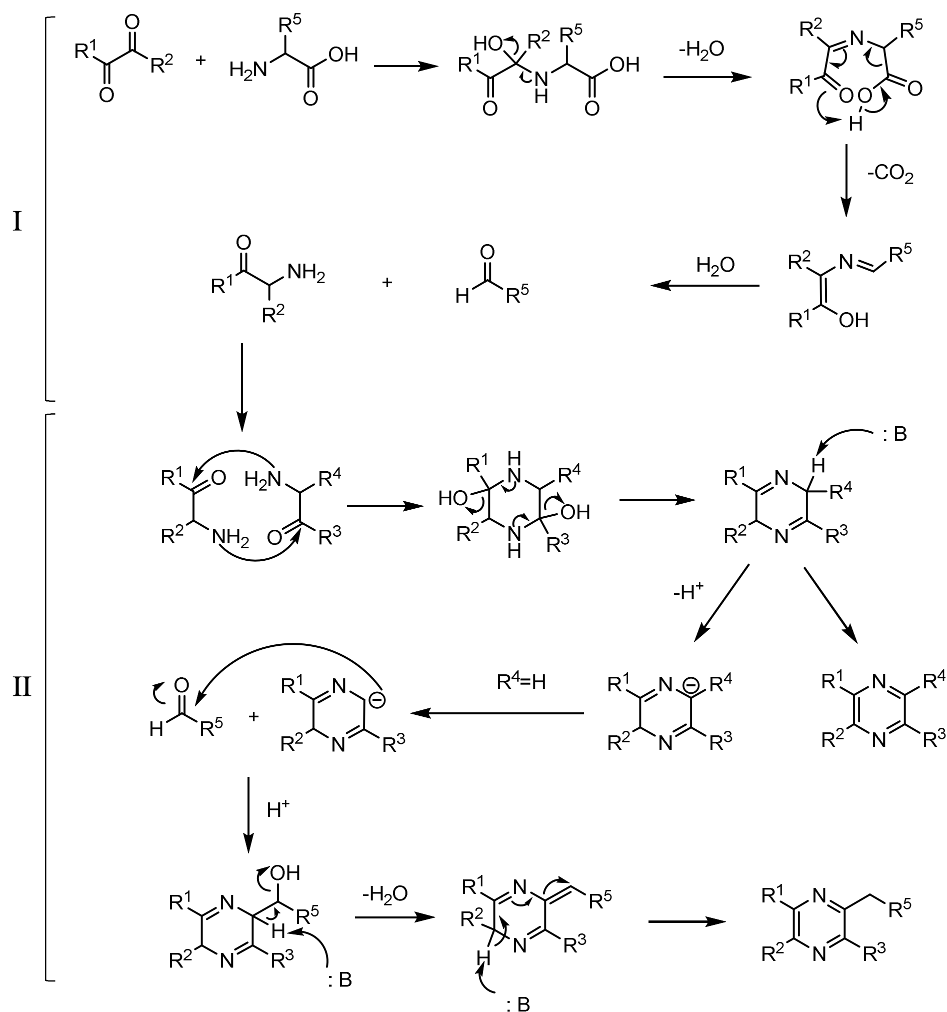


Figure 28. Mechanism of pyrazine formation via Maillard reaction (Scalone *et al.*, 2015).

Something similar has been described for the formation of pyridines (Karademir *et al.*, 2019). These compounds have been found in a wide range of processed foods, although to a lower extent than pyrazines (Maga, 1981). When alkadienals are heated in presence of asparagine, the formation of pyridines is observed (Zamora *et al.*, 2009).

Analogously to that observed in their reaction with amino acids, this tendency of reactive carbonyls to produce heterocyclic derivatives is mostly unknown. In fact, it is not known what happens when reactive carbonyls are heated in the presence of ammonia. Furthermore, to the best of our knowledge, the role of other nucleophiles in the cyclization ability of reactive carbonyls has not been studied so far. This may be particularly important for reactions involving creatinine. Murkovic's group showed that creatinine is added to phenylacetaldehyde (Murkovic *et al.*, 1999; Zöchling & Murkovic, 2002). Furthermore, when Zamora *et al.* (2014) heated this adduct in the presence of ammonia and formaldehyde, the heterocyclic aromatic amine (PhIP) was obtained, therefore suggesting that reactive carbonyls might be involved in the formation of heterocyclic aromatic amines.

As a continuation of those studies, this Ph.D thesis investigates the ability of reactive carbonyls to produce cyclic derivatives (including flavour-relevant heterocyclic compounds), and the effect that the presence of creatinine has in these reactions, which can play a major role in the formation of heterocyclic aromatic amines with the structure of aminoimidazoazarene. These studies should provide the basis to understand the formation of all these kinds of compounds so that the formation of beneficial compounds can be promoted and the generation of toxic compounds inhibited.

2. AIMS OF STUDY

In spite of the more than four decades elapsed from their discovery, the origin of most heterocyclic aromatic amines is still unknown. Nevertheless, the recent proposal of a formation pathway for PhIP suggests that reactive carbonyls might be playing a role in the formation of heterocyclic aromatic amines, especially in those with the structure of aminoimidazoazarene. This would be a consequence of an, at present unknown, tendency of reactive carbonyl compounds to cyclise and oligomerise under appropriate conditions. The general objective of this study is to clarify the contribution of reactive carbonyls to the formation of heterocyclic derivatives, including the formation of both, flavours and heterocyclic aromatic amines, to provide tools that allow the control of their presence in foods.

To carry out this general objective, different specific objectives are proposed:

1. The investigation of the role of cyclizations of reactive carbonyls on the formation of food flavours, in particular the formation of alkylpyridines.
2. The study of the formation of 3-hydroxypyridines in foods by lipid-derived reactive carbonyls.
3. The examination of the role of the products of Maillard reaction, in particular 5-hydroxymethylfurfural, on the formation of 3-hydroxypyridines.
4. The search for the precursors and reaction pathways involved in the formation of 2-amino-3,4-dimethylimidazo(4,5-*f*)quinoline (MeIQ).
5. The identification of the carbonyl compounds responsible for the formation of 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ) as well as the reaction pathway by which this heterocyclic aromatic amine is formed.
6. The determination of precursors responsible for the formation of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) as well as its formation pathway.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Commercial products

Many different products were employed in these studies. Some of them were purchased from commercial sources and others were prepared in the laboratory.

Commercial products had always the highest available grade, usually analytical grade. Distributors included Sigma-Aldrich (St. Louis, MO, USA), Alfa Aesar (Haverhill, MA), TCI (Tokyo, Japan), and Merck (Darmstadt, Germany).

Different lipid derivatives were employed, including both unoxidized and oxidized lipids. The employed unoxidized lipids were linoleic acid, linolenic acid, and their methyl esters, and menhaden and linseed oils. In addition, many commercial reactive carbonyls produced in the lipid oxidation pathway were assayed. They included alkanals (ethanal, propanal, butanal, and hexanal), 2-alkenals (2-hexenal and 2-heptenal), 4-alkenals (4-pentenal), 2,4-alkadienals (2,4-hexadienal, 2,4-heptadienal, 2,4-octadienal, 2,4-nonadienal, and 2,4-decadienal), ketones (3,5-heptadien-2-one and 6-methyl-5-hepten-2-one), and dialdehydes (glyoxal, malondialdehyde, and fumaraldehyde). Among carbohydrates, monosaccharides (glycolaldehyde, arabinose, rhamnose, ribose, glucose, fructose, and galactose), disaccharides (sucrose and lactose), and reactive carbonyls derived from them [furfural, 5-hydroxymethylfurfural (HMF), and 2-acetylfuran] were also employed. Finally, as amino compounds, ammonia, ammonium chloride, glutamine, urea, and creatinine, were used.

Commercial products also included standards. The followed pyridines were used: 2-Methylpyridine, 3-methylpyridine, 4-methylpyridine, 2-ethylpyridine, 3-ethylpyridine, 2-pentylpyridine, 2,5-dimethylpyridine, 2,6-dimethylpyridine, 5-ethyl-2-methylpyridine, 3-acetylpyridine, 3-hydroxy-2-methylpyridine, 3-hydroxypyridine, and 2-hydroxymethyl-5-hydroxypyridine. In addition,

standards of PhIP, IQ, MeIQ, and MeIQx (obtained from Toronto Research Chemicals, North York, ON, Canada) were also employed.

Multi-floral honey and sugarcane honey or sugarcane syrup (a traditional plant syrup, namely 'miel de caña') were purchased from a local supermarket.

All solvents used in these studies (hexane, ethyl acetate, acetone, acetonitrile, chloroform, tetrahydrofuran, and toluene) were analytical grade and were purchased from Merck (Darmstadt, Germany). Citric acid (99.5%), boric acid, sodium hydroxide (99%), and the sodium phosphate used to prepare the different buffers, were also obtained from Merck. Silica 60 (thickness of 0.063-0.2 mm) for column chromatography was purchased from Macherey Nagel (Gmbh, Düren, Germany).

3.1.2. Syntheses of lipid hydroperoxides

Two lipids hydroperoxides and their methyl esters were employed in these studies. All of them were synthesised by following a previously described procedure (Zamora & Hidalgo, 2008). 13-Hydroperoxy-9,11-octadecadienoic acid (13-LOOH) and methyl 13-hydroperoxyoctadeca-9,11-dienoate (MeLOOH) were prepared by oxidation of linoleic acid with soya lipoxygenase and later esterification with diazomethane. Analogously, 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-LnOOH) and methyl 13-hydroperoxyoctadeca-9,11,15-trienoate (MeLnOOH) were obtained from linolenic acid. The followed procedure is described briefly.

A solution of the corresponding fatty acid (2.1 mmol) in absolute ethanol (1 mL) was added to 20 mL water and sonicated until permanent emulsion. Then, the emulsion was treated with 2 N NaOH until the solution was cleared and taken to 200 mL with 50 mM sodium borate, pH 9. Sodium borate buffer (500 mL of 50 mM), pH 9, was oxygenated for 15 min at 0 °C by bubbling in a slow stream of oxygen. After this time, 292 units of lipoxygenase (obtained from sigma),

dissolved in 10 mL of 50 mM sodium borate buffer, pH 9, and the fatty acid solution was added. The lipoxygenation was carried out for 10 min at 0 °C in the presence of oxygen. The reaction was stopped by lowering the pH to 3 and extracting the hydroperoxide with diethyl ether (5 × 100 mL). The organic layers were combined, dried over anhydrous sodium sulphate and evaporated. 13-LOOH and 13-LnOOH were obtained in that way. To obtain their methyl esters, diazomethane was employed. In this case, samples had to be purified by column chromatography on silica gel using hexane/diethyl ether (7:3) as the eluent. The separation was controlled by thin layer chromatography, 13-LOOH, 13-LnOOH, MeLOOH, and MeLnOOH (**Figure 29**) were obtained chromatographically pure.

Additional confirmations of identity and purity were obtained by 1D and 2D NMR. ^{13}C NMR ($CDCl_3$, 75.4 MHz) of MeLOOH: δ (ppm) 174.49 (C1), 133.96 (C9), 131.23 (C12), 130.11 (C11), 127.51 (C10), 86.85 (C13), 51.54 (OCH_3), 34.06 (C2), 32.49 (C14), 31.72 (C16), 29.35, 29.03, 29.00, 28.87 (C4-C7), 27.69 (C8), 24.98 (C15), 24.85 (C3), 22.51 (C17), and 14.04 (C18). ^{13}C NMR ($CDCl_3$, 75.4 MHz) of MeLnOOH: δ (ppm) 174.53 (C1), 134.35 and 134.11 (C9 and C16), 130.37 (C12), 130.20 (C11), 127.50 (C10), 123.09 (C15), 86.21 (C13), 51.56 (OCH_3), 34.07 (C2), 30.58 (C14), 29.34, 29.03, 29.00, and 28.87 (C4-C7), 27.69 (C8), 24.85 (C3), 20.70 (C17), and 14.12 (C18) (Zamora & Hidalgo, 2008).

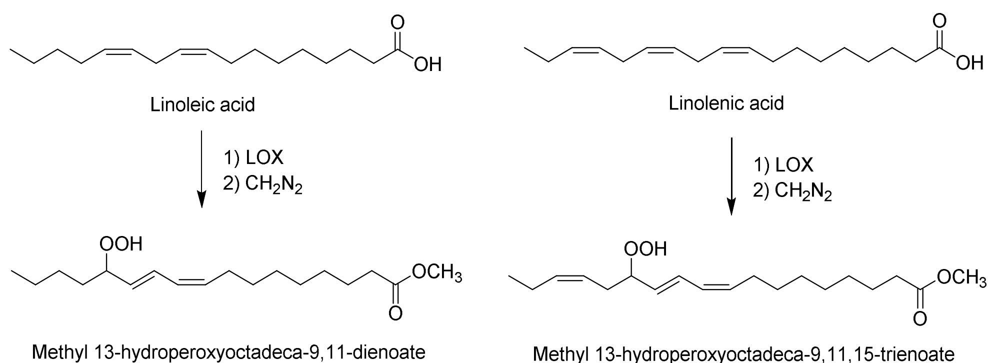


Figure 29. Syntheses of methyl 13-hydroperoxyoctadeca-9,11-dienoate (MeLOOH) and methyl 13-hydroperoxyoctadeca-9,11,15-trienoate (MeLnOOH). Abbreviations: LOX, lipoxygenase.

3.1.3. Syntheses of 4-hydroxy-2-alkenals

Two 4-hydroxy-2-alkenals were employed in this study. They were 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE). 4-Hydroxy-2-nonenal (**Figure 30**) was synthesised following the procedure of Gardner *et al.* (1992). Briefly, to a solution of 3-nonenol (35 mmol) in 14 mL dichloromethane, a 1.2 molar excess of 80-85% 3-chloroperoxybenzoic acid (54 mmol) was added. The solution was kept at room temperature for 1 h after which 2 mL of 10% $NaHCO_3$ was added with vigorous stirring for 45 min. The organic layer, containing 3,4-epoxynonanol, was oxidised with periodinane (38 mmol) in dichloromethane (150 mL). The oxidation mixture was stirred for 30 min at room temperature, and then, 550 mL of diethyl ether and 300 mL of 1.3 M NaOH were added with vigorous stirring for 10 min. The aqueous layer was removed, and the organic phase was washed with 300 mL of H_2O. Evaporation of solvent afforded crude 4-hydroxy-2-nonenal, which was obtained chromatographically pure by column chromatography on silica gel using hexane:diethyl eter (2:1) as a solvent. The separation was controlled by thin layer chromatography.

Its identity was confirmed by ^1H NMR. ^1H NMR ($CDCl_3$, 300 MHz): δ 0.80 (br t, 3H), 1.4 (m, 8H), 2.58 (br s, 1H), 4.40 (m, 1H), 6.26 (ddd, J = 1.0, 7.0, 15.5, Hz, 1H), 6.80 (dd, J = 4.0, 15.5 Hz, 1H), 9.56 (d, J = 8.0 Hz, 1H).

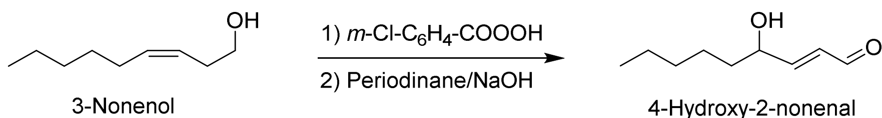


Figure 30. Synthesis of 4-hydroxy-2-nonenal (HNE).

4-Hydroxy-2-hexenal (**Figure 31**) was prepared from 3(*Z*)-hexenol according to Hidalgo & Zamora (2019) following a procedure similar to that of Gardner *et al.* (1992), but with significant modifications. Briefly, a solution of 3(*Z*)-hexenol (50 mmol) in 140 mL of dichloromethane was treated slowly with 3-

chloroperoxybenzoic acid (9.3 g), and the obtained mixture was stirred for 1 h at room temperature. After this time, 10% potassium bicarbonate (140 mL) was added and the mixture was stirred for 2 min. The organic layer was then collected and dried over sodium sulphate. The obtained solution, which contained produced 3,4-epoxyhexanol, was slowly added to a solution of periodinane (16 g in 150 mL of dichloromethane), and the obtained mixture was stirred under dark for 30 min at room temperature. Then, 500 mL of diethyl ether and 230 mL of 1.3 M NaOH were added. The solution was vigorously stirred for 2 min, and the organic layer was collected, dried over sodium sulphate, and taken to dryness. The residue was fractionated by column chromatography on silica gel using mixtures of hexane and diethyl ether as the eluent. The separation was controlled by thin layer chromatography. Identity and purity of obtained HHE were confirmed by means of one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) and gas chromatography–mass spectrometry (GC–MS).^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.02 (br t, 3H), 1.68 (m, 8H), 4.40 (m, 1H), 6.34 (ddd, *J* = 1.6, 7.9, 15.7 Hz, 1H), 6.81 (dd, *J* = 4.7, 15.7 Hz, 1H), 9.60 (d, *J* = 7.9 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 9.43 (CH_3), 29.47 ($\underline{C}H_2CH_3$), 72.27 ($\underline{C}HOH$), 130.89 ($\underline{C}HCHO$), 158.68 ($\underline{C}HCHOH$). MS, *m/z* (%, ion structure): 114 (0.1, M^+), 96 (0.3, ethylfuran), 85 (60, $M^+ - CH_3CH_2$ or CHO), 67 (6, 96 – CH_3CH_2), 57 (100, 96 – C_3H_3), and 55 (12, $M^+ - CH_3CH_2CHOH$).

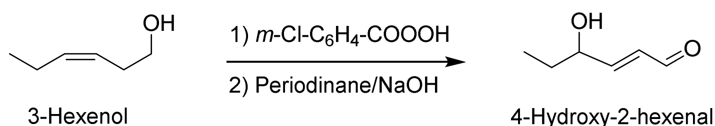


Figure 31. Synthesis of 4-hydroxy-2-hexenal (HHE).

3.1.4. Syntheses of 4-oxo-2-alkenals

Two 4-oxo-2-alkenals were employed in this study: 4-Oxo-2-hexenal and 4-oxo-2-nonenal. Both compounds were prepared by ring opening of the corresponding 2-alkylfuran: 2-Ethylfuran and 2-pentylfuran, respectively (**Figure 32**) (Hidalgo *et al.*, 2018a).

Briefly, *N*-bromosuccinimide (10.7 mmol) and pyridine (10 mL) were added to a solution of 2-alkylfuran (16.6 mmol) in 11 mL of tetrahydrofuran/acetone/water (5:4:2) in an ice bath. The reaction mixture was stirred for 1 h at this temperature and, then, kept on stirring at room temperature for 2 h. After this time, the mixture was diluted with 10 mL of water and extracted three times with 50 mL of chloroform. The combined chloroformic extracts were washed successively with hydrochloric acid and water in order to remove pyridine, then dried over anhydrous sodium sulphate, and finally concentrated under vacuum. The residue was fractionated by column chromatography on silica gel 60 (230-400 mesh; Macherey-Nagel) using mixtures of hexane and ethyl acetate as eluent. The separation was controlled by thin layer chromatography. 4-Oxo-2-alkenals were obtained chromatographically pure. Confirmations of identity and purity were obtained by GC-MS by comparison with the retention times and mass spectra of authentic standard previously prepared in the laboratory.

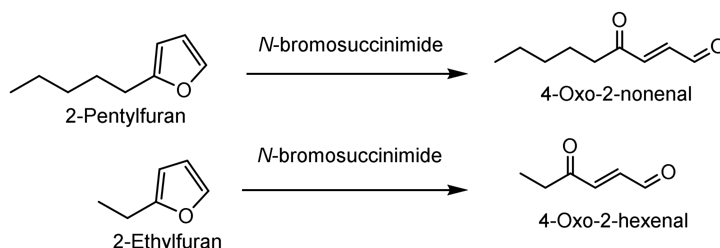


Figure 32. Syntheses of 4-oxo-2-nonenal and 4-oxo-2-hexenal.

3.1.5. Syntheses of 4,5-epoxy-2-alkenals

Three 4,5-epoxy-2-alkenals (**Figure 33**) were employed in this study. 4,5-Epoxy-2-hexenal, 4,5-epoxy-2-heptenal, and 4,5-Epoxy-2-decenal were prepared by epoxidation of 2,4-hexadienal, 2,4-heptadienal, and 2,4-decadienal, respectively, with 3-chloroperoxybenzoic acid according to the procedure of Zamora *et al.* (2017).

Briefly, 3-chloroperoxybenzoic acid (25 mmol) was dissolved in chloroform (175 mL), washed three times with 100 mL of buffer (0.2 M Na_2HPO_4 adjusted to pH 7.5 with 0.1 M citric acid) followed by three 100 mL portions of water, and dried with anhydrous sodium sulphate. This solution was added slowly to a solution of 2,4-alkadienal (39.9 mmol) in chloroform (30 mL), which was stirred at room temperature overnight and, finally, washed three times with 100 mL of buffer (0.2 M Na_2HPO_4 adjusted to pH 7.5 with 0.1 M citric acid), and, finally, followed by three extractions with 100 mL of water. The organic solution was dried over anhydrous sodium sulphate and concentrated under vacuum. The residue was fractionated by column chromatography on silica gel 60 (230-400 mesh; Macherey-Nagel) using mixtures of hexane and acetone as eluent. The separation was controlled by thin layer chromatography. 4,5-Epoxy-2-alkenals were obtained chromatographically pure. Confirmations of identity and purity were obtained by gas chromatography-mass spectrometry (GC/MS) comparison was carried out with retention times and mass spectra with authentic standards prepared previously in the laboratory.

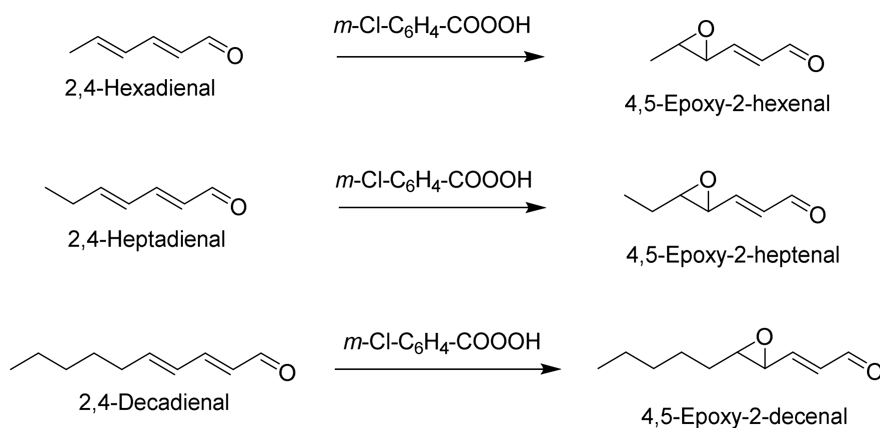


Figure 33. Syntheses of 4,5-epoxy-2-alkenals.

3.2. Analytical instrumentation

3.2.1. Gas chromatography-mass spectrometry (GC-MS)

Two different instruments were employed for GC-MS analyses. Instrument no. 1 was an Agilent 6890 GC Plus coupled to an Agilent 5973 MSD (mass selective detector, quadrupole type). Instrument no. 2 was an Agilent 7820A GC coupled to an Agilent 5977 MSD. In each chromatograph, a 30 m length × 0.25 mm i.d. × 0.25 μm coating thickness HP5-MS capillary column was used. Samples (1 μL) were injected in the pulsed splitless mode. Helium (1 mL/min at constant flow) was employed as carrier gas. The following working conditions were used: Injector, 250 °C; transfer line to mass selective detector, 280 °C; electron ionization (EI), 70 eV; ion source temperature, 230 °C; and mass range, 28–550 amu. Different oven programs were employed depending on the samples to be analysed. They included:

- Oven program 1: From 40 °C (3 min) to 200 °C at 20 °C/min, and then held at 200 °C for 1 min.
- Oven program 2: From 80 °C (1 min) to 140 °C at 20 °C/min, then to 300 °C at 50 °C/min, and finally held at 300 °C for 4 min.

3.2.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

An Agilent liquid chromatography system (1200 Series), coupled to a triple quadrupole API 2000 mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used. Reaction mixtures were fractionated on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm) from Agilent. An electrospray ionization interface in positive ionization mode (ESI^+) was used. Mobile phase was delivered at 0.5 mL/min using a gradient of eluents A (30mM ammonium formiate) and B (acetonitrile). The employed gradient was (% B in A): From 5 to 60% (15 min), from 60 to 100% (5 min), from 100 to 5% (1 min), and finally isocratic reconditioning for 5 min.

Mass spectrometric acquisition was performed using multiple reaction monitoring (MRM). The conditions for the mass detector were: Nebulizer gas (synthetic air), curtain gas (nitrogen), heater gas (synthetic air), and collision gas (nitrogen) were set at 45, 35, 50, and 5 (arbitrary units), respectively; the heater gas temperature was set at 350 ºC; and the electrospray capillary voltage was 5.5 kV.

3.2.3. Nuclear magnetic resonance spectroscopy (NMR)

Most 1D and 2D NMR spectra were obtained in a Bruker Advance III spectrometer operating at 500 MHz for protons. Unless otherwise indicated, samples were dissolved in $CDCl_3$ and acquired using a 30º flip angle pulse for protons with a delay of 1 s. ^{13}C NMR were acquired using a 30º flip angle and power-gated pulse decoupling. A delay of 2 s was used. As a help for structural determination DEPT, COSY, HSQC, and HMBC spectra were obtained.

3.2.4. Other equipment

Other used equipment included rotary evaporator (Büchi R210, Büchi Labotechnik AG, Flawil, Suiza), oven (Memmert ULE 400 with forced convection, Schwabach, Germany), block heater (Grant QBH2, Cambridge, UK), vortex (Heidolph Reax 2000, Schwabach, Germany), vacuum pump (Vacuubrand, Wertheim, Germany), bath (Selecta, Univeba, Barcelona, Spain), magnetic stirrer hotplate (Gallenkamp, Loughborough, UK), centrifuge (Hettich Universal 16R, Germany), heating/stirrer module for solvent evaporation with nitrogen (Thermo Scientific Reacti-Therm III TS-18823, Waltham, USA), fraction collector (LKB 7000 ultrorac, Stockholm, Sweden), fraction collector (Gilson Model 203, Middleton, WI), analytical precision balances (Sartorius, Goettingen, Germany), and portable gas analyser (Dansensor, Ringsted, Denmark).

3.3. Studied systems

3.3.1. Formation of alkyl- and dialkyl-pyridines

The general procedure was similar to that previously employed for model studies on acrylamide formation (Hidalgo *et al.*, 2009). Briefly, mixtures of the reactive carbonyl (or two reactive carbonyls if needed) (50 μmol of each one in 50 μL of methanol), the ammonia-producing compound (10 μmol in 45μL of water) [or 50 μmol in 50 μL of water in the case of alanine], 30 μL of 0.3 M sodium phosphate, pH 6.5, and 50 μL of water, were singly homogenized with 0.063–0.20 mm silica gel (300 mg) (Macherey-Nagel, Düren, Germany).

To confirm reaction patways, some reaction mixtures were heated in the presence of deuterated water, which substitute H_2O in the general recipe. As a consequence of this, deuterated pyridines were produced. These pyridines were studied by GC-MS analogously to the above described for non-deuterated pyridines.

Samples were heated at 180 ºC in closed test tubes for 1 h. After cooling, 700 μL of methanol and 30 μL of the internal standard solution (19 μmol of methyl heptanoate in 1 mL of methanol) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was analysed by GC-MS using the oven temperature programme no. 1.

Reactions involving menhaden oil were carried out similarly, although some modifications were required. Briefly, mixtures of the oil (1 g), glutamine (30 μmol in 150 μL of water), 25 μL of methanol, and 30 μL of 0.3 M sodium phosphate, pH 6.5, were heated at 180 ºC in closed test tubes for 1 h. After cooling, 700 μL of acetonitrile and 30 μL of the internal standard solution (19 μmol of methyl heptanoate in 1 mL of methanol) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was analysed by GC-MS using the oven temperature programme no. 1.

3.3.2. Formation of 3-hydroxypyridines

Mixtures of the reactive carbonyl (20 μmol in 20 μL of methanol), the ammonia-producing compound (5 μmol in 50 μL of water), and 30 μL of 0.3 M buffer (sodium citrate pH 3–6, sodium phosphate pH 6–8, or sodium borate pH 8–10) were singly homogenized with 200 mg of 0.063–0.020 mm silica gel (Macherey-Nagel, Düren, Germany),.

Samples were heated in closed test tubes for the indicated times and temperatures. After cooling, 1 mL of methanol and 20 μL of the internal standard solution (13 mg of 1-octadecanol in 25 mL of methanol) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was evaporated to dryness and the residue was successively treated with 200 μL of *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 30 min at 60 ºC, and studied by GC-MS using the oven temperature programme no. 2.

3.3.3. Formation of 3-hydroxypyridines in oxidized oils

The procedure was analogous to that described in section 3.3.2, although small changes were needed. Briefly, mixtures of the oil (50 mg) and 30 μL of 0.3 M sodium phosphate, pH 8, were singly homogenized with 200 mg of 0.063–0.020 mm silica gel (Macherey-Nagel, Düren, Germany).

These mixtures were incubated in closed test tubes under air for 5 days at 60 ºC. After cooling for 10 min at room temperature and another 10 min at 4 ºC, glutamine (5 μmol in 50 μL of water) was added and samples were heated at 100 ºC for 22 h. At the end of the heating time, samples were cooled, and 1 mL of methanol and 20 μL of the internal standard solution (13 mg of 1-octadecanol in 25 mL of methanol) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was evaporated to dryness and

the residue was derivatizated with *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA), and studied by GC-MS, using the oven temperature programme no. 2.

3.3.4. Conversion of 2-oxofurans into 3-hydroxypyridines

Mixtures of the 2-oxofuran (10 μmol in 20 μL of methanol), the ammonia-producing compound (30 μmol in 50 μL of water), and 30 μL of 0.3 M buffer (sodium citrate pH 3–6, sodium phosphate pH 6–8, or sodium borate pH 8–10) were singly homogenized with 200 mg of 0.063–0.200 mm silica gel (Macherey-Nagel, Düren, Germany).

Samples were heated in closed test tubes for the indicated times and temperatures. After cooling, 1 mL of methanol and 20 μL of the internal standard solution (13 mg of 1-octadecanol in 25 mL of methanol) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was collected, evaporated to dryness, and the residue was successively treated with 200 μL of *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and heated for 30 min at 60 ºC. The formation of the corresponding 3-hydroxypyridine was studied by GC-MS, using the oven temperature programme no. 2.

3.3.5. Formation of 3-hydroxypyridines in honeys

Honeys (1 g) were heated in closed test tubes for 0–10h at either 60 ºC or 100 ºC. After cooling, 100 μL of water was added and the mixture was stirred for 1 min. Then, 4 mL of acetonitrile and 20 μL of the internal standard solution (13 mg of 1-octadecanol in 25 mL of methanol) were added. Mixtures were successively stirred for 1 min, sonicated for 10 min, stirred for 1 min, and the acetonitrile layer was decanted and taken to dryness. The residue was treated with 300 μL of *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and heated for 30 min at 60 ºC. The formation of the corresponding 3-hydroxypyridines was studied by GC-MS using the oven temperature programme no. 2.

3.3.6. Reaction of reactive carbonyls, creatinine, and ammonia-producing compounds. Formation of MeIQ

Mixtures of the reactive carbonyl(s) (80 µmol in 20 µL of methanol), creatinine (20 µmol in 30 µL of water), the ammonia-producing compound (10 µmol in 45 µL of water), and 30 µL of 0.3 M of sodium phosphate, pH 6.5, were singly homogenized with 0.063–0.200 mm silica gel (300 mg).

Reaction mixtures were heated at 180 ºC in closed test tubes for 1 h. After this period, samples were cooled and 2 mL of ethyl acetate and 30 µL of the internal standard solution (6.45 mg of caffeine in 10 mL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate) were added. Suspensions were stirred for 1 min and centrifuged for 5 min at 2000 *g*. The supernatant was isolated and taken to dryness with nitrogen. The resulting residue was extracted with 200 µL of a 1:1 mixture of 30 mmol/L ammonium formiate and acetonitrile, and the extract was studied by LC-MS/MS.

3.3.7. Reaction of acrolein, crotonaldehyde, creatinine, and glutamine. Formation of IQ

This procedure was analogous to that described in section 3.3.6. However, this time the reaction was carried out in two consecutive steps.

Firstly, mixtures of acrolein (20 µmol in 20 µL of methanol), creatinine (30 µmol in 45 µL of water), and 30 µL of 0.3 M sodium phosphate, pH 6.5, were singly homogenized with 0.063–0.200 mm silica gel (300 mg). Reaction mixtures were incubated overnight in closed test tubes at 25 ºC. After this time, crotonaldehyde (10 µmol in 20 µL of methanol) and glutamine (10 µmol in 45 µL of water) were added, tubes were closed, and reaction mixtures were heated at 180 ºC for 1 h. After this period, samples were treated as described in section 3.3.6, and the formed heterocyclic aromatic amines were identified and determined by LC-MS/MS.

3.3.8. Reaction of reactive carbonyls and creatinine. Formation of MeIQx

The employed procedure was analogous to that described in sections 3.3.6 and 3.3.7. Briefly, mixtures of the reactive carbonyl(s) (50 µmol in 20 µmol of methanol), creatinine (50 µmol in 60 µL of water), 30 µL of 0.3 M sodium phosphate, pH 6.5, and 40 µL of water were singly homogenized with 0.063–0.200 mm silica gel (300 mg).

Samples were heated at 180 ºC in closed test tubes for 1 h. Heated samples were treated as described in section 3.3.6, and formed MeIQx was identified and determined by LC-MS/MS.

3.3.9. Reaction of acrolein-producing compounds and creatinine. Formation of MeIQx

Model reactions were carried out analogously to section 3.3.8, but acrolein was replaced by acrolein-producing compounds. Assayed compounds were those that previous studies (Stevens & Maier, 2008) have shown to be acrolein-producing compounds.

Studied compounds included carbohydrates (glucose, fructose, and ribose), fats (linseed oil, menhaden oil, and 13-hydroperoxide of linoleic acid), and amino acids (threonine and methionine).

For linseed and menhaden oils the experimental procedure was slightly different to that employed for the other compounds. Thus, these oils were oxidized in a previous step to their reaction with creatinine to promote acrolein formation. The procedure employed for these oils was as follows: Mixtures of the oil (50 mg), 300 mg of silica gel, and 30 µL of 0.3 M of sodium phosphate, pH 6.5, were incubated for 4 days at 60 ºC in closed test tubes. After that time, 50 µmol of creatinine was added and mixtures were heated at 180 ºC for 1 h. After cooling, reaction mixtures were treated as described in section 3.3.6, and studied by LC-MS/MS.

3.4. Identification and quantification of studied compounds

3.4.1. Identification and quantification of alkylpyridines

Identification of most pyridines was carried out by comparison with retention times, mass spectra, and co-elution with authentic standards. When these standards were not available, tentative identifications were carried out on the basis of their mass spectra.

Quantification of pyridines was carried out by preparing standard curves of these compounds. Seven concentration levels (0–10 µmol) for each pyridine was used and the procedure described in the section 3.3.1 was followed. Compound content was directly proportional to compound/internal standard area ratio ($r^2 > 0.98$, $p < 0.001$). RSD was always < 10%.

When the commercial standards were not available, such as in the case of 2-propylpyridine or 2-butylpyridine, the concentration of the compound was estimated by using the calibration curve of the compound with the most similar chemical structure. Thus, the calibration curve of 2-ethylpyridine was used for 2-propylpyridine, and the calibration curve of 2-pentylpyridine was employed for 2-butylpyridine.

3.4.2. Identification and quantification of 3-hydroxypyridines

Each compound was identified according to its mass spectrum, retention index, and by co-elution with authentic standards (when available).

The M^+ and the (M^+–methyl) ions of the 3-hydroxypyridine and the 3-hydroxy-2-alkylpyridines were employed for identification purposes. The second one, which was the base ion for most of studied pyridines, was used for quantification purposes.

Linear retention indexes for the different hydroxypyridines increased linearly ($r^2 = 0.994$, $p < 0.0001$) as a function of the chain length of the alkyl substituent as observed in **Figure 34**.

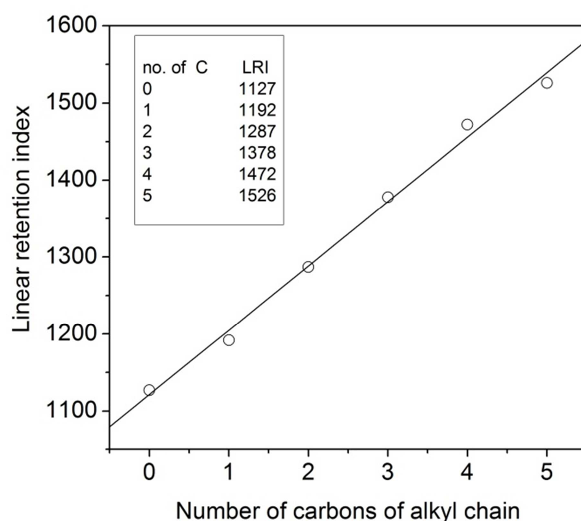


Figure 34. Linear retention indexes (LRI) of the trimethylsilyl derivatives of the hydroxyalkylpyridines.

Mass spectra of the trimethylsilyl derivatives of the different studied hydroxypyridines are shown in **Figure 35.** Their mass spectra, relative intensities, and ion structures for the different ions were:

3-Hydroxypyridine, *m/z* (relative intensity, ion structure): 167 (32, M^+), 152 (100, M^+–methyl), 73 (17, trimethylsilyl).

3-Hydroxy-2-methylpyridine, *m/z* (relative intensity, ion structure): 181 (40, M^+), 166 (100, M^+–methyl), 73 (45, trimethylsilyl).

2-Ethyl-3-hydroxypyridine, *m/z* (relative intensity, ion structure): 195 (5, M^+), 180 (100, M^+–methyl), 166 (23, M^+–ethyl), 165 (22, M^+–ethane), 73 (54, trimethylsilyl).

3-Hydroxy-2-propylpyridine, *m/z* (relative intensity, ion structure): 209 (5, M^+), 194 (100, M^+–methyl), 166 (31, M^+–propyl), 165 (26, M^+–propane), 73 (46, trimethylsilyl).

2-Butyl-3-hydroxypyridine, *m/z* (relative intensity, ion structure): 223 (8, M^+), 208 (100, M^+–methyl), 180 (10, M^+–propyl), 166 (40, M^+–butyl), 165 (22, M^+–butane), 73 (54, trimethylsilyl).

3-Hydroxy-2-pentylpyridine, *m/z* (relative intensity, ion structure): 237 (1, M^+), 222 (6, M^+–methyl), 208 (14, M^+–ethyl), 194 (23, M^+–propyl), 181 (42, M^+–butene), 180 (41, M^+– butyl), 166 (100, M^+–pentyl), 73 (63, trimethylsilyl).

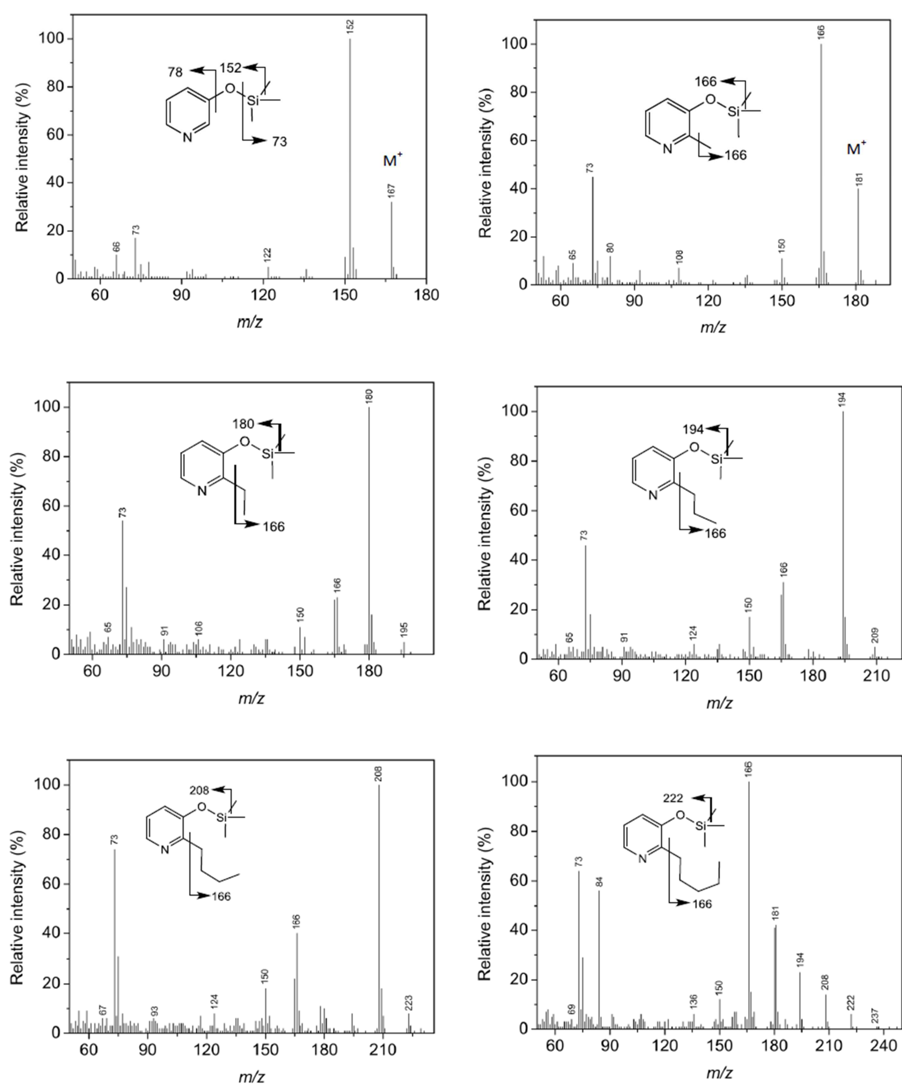


Figure 35. Mass spectra of the trimethylsilyl derivatives of the studied hydroxyalkylpyridines.

In studies involving the formation of 2-hydroxymethyl-5-hydroxypyridine from HMF, the identification of both compounds was carried out by comparison of retention indexes and mass spectra, and by co-elution with authentic standards which were derivatizated analogously. The M^+ (*m/z* 269 and 198) and $M^+–1$ (*m/z* 254 and 183) ions of 2-hydroxymethyl-5-hydroxypyridine and HMF, respectively, were employed for identification purposes. The second one was used for quantification purposes.

Quantification of all 3-hydroxypyridines and HMF in model and food systems were carried out by preparing standard curves of all these compounds in the corresponding assayed systems and following the whole procedures described in sections 3.3.2, 3.3.3, 3.3.4, and 3.3.5 (without heating). Different concentration levels of each compound were used to prepare the standard curves. Compound contents were directly proportional to compound/internal standard area ratio ($r^2 > 0.98$, $p < 0.001$). The coefficients of variation at the different assayed concentrations were < 10%. When a standard of hydroxypyridine derivative was not available, the quantification of this compound was carried out by using the calibration curve of the available compound with the highest structural similarity.

3.4.3. Identification and quantification of heterocyclic aromatic amines

Heterocyclic aromatic amines (MeIQ, IQ, and MeIQx) were determined by LC-MS/MS in the samples prepared as described in sections 3.3.6, 3.3.7, 3.3.8, and 3.3.9, respectively. Three transitions were acquired for the identification of each compound and caffeine was employed as internal standard. To establish the appropriate MRM conditions, the mass spectrometric conditions of authentic standards were optimized by using infusion with a syringe pump. Precursor and product ions used for qualification and quantification purposes, and operating conditions for each compound are summarized in **Table 6**. The first transition was employed for quantification and the others were used to confirmation purposes.

Quantitative determinations of heterocyclic aromatic amines were carried out by preparing calibration curves of authentic standards and following the whole procedures described in sections 3.3.6, 3.3.7, 3.3.8, and 3.3.9. For each curve, six concentration levels of the different heterocyclic aromatic amines were used. Heterocyclic aromatic amine content was directly proportional to the heterocyclic aromatic amine/internal standard area ratio ($r^2 > 0.986$, $p < 0.0001$). The coefficients of variation at the different concentrations were always < 15%.

Table 6. MRM transitions employed for the detection of IQ, MeIQ, and MeIQx.

HAAs	Transition	DP	FP	EP	CEP	CE	CXP
IQ	199.5 → 185.1	46	370	12	16	37	6
	199.5 → 157.1	46	370	12	16	51	6
	199.5 → 158.1	46	370	12	16	45	6
MeIQ	213.9 → 199.1	46	370	10	16	37	6
	213.9 → 198.1	46	370	10	16	45	8
	213.9 → 171.2	46	370	10	16	55	8
MeIQx	215.0 → 200.1	41	370	10	16	37	6
	215.0 → 132.1	41	370	10	16	53	4
	215.0 → 77.0	41	370	10	16	77	2
Caffeine	195.2 → 138.0	31	350	10.5	12	27	6
(IS)	195.2 → 110.1	31	350	10.5	12	33	4
	195.2 → 42.1	31	350	10.5	12	55	4

Abbreviations: DP, declustering potential; FP, focusing potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

3.5. Statistical analyses

All data given are mean ± SD values of, at least, three independent experiments. Analysis of variance was employed to compare group differences. When significant F values were obtained, group differences were evaluated by the Tukey test (Snedecor & Cochran, 1980). Statistical comparisons were carried out using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

4. RESULTS

This section is divided into two parts. The first part is dedicated to the role that cyclization and oligomerization reactions of reactive carbonyls have in the formation of food flavours, in particular pyridines. The second part is dedicated to cyclization reactions of reactive carbonyls in the presence of creatinine.

4.1. Cyclization and oligomerization of reactive carbonyls: A route for the production of pyridines in foods

Pyridines are produced as a consequence of food processing and they contribute to the characteristic sensory properties of food products, although they are not major food flavours. This section describes the cyclizations and ring-expansions of reactive carbonyls (2-alkenals, 2,4-alkadienals, 4,5-epoxy-2-alkenals, HMF, furfural, and 2-acetylfuran) in the presence of ammonia-producing compounds.

4.1.1. Cyclization and oligomerization of reactive carbonyls in the presence of ammonia-producing compounds

4.1.1.1. *Formation of pyridines by oligomerization of crotonaldehyde in the presence of ammonia-producing compounds*

When crotonaldehyde was heated in presence of amino compounds, the formation of pyridines was observed (**Table 7**). As can be observed, four pyridines were produced: 2-Methyl, 3-methyl-, 2,5-dimethyl, and 5-ethyl-2-methyl-pyridine. The same four pyridines were always produced independently of the employed amino compound (as expected, pyridines were not produced when the amino compound was absent, data not show). In addition, the highest amounts of pyridines were produced when ammonia was added. This suggested that the contribution of amino compounds was related to their ability of producing ammonia.

Reaction yields were quite high. When either ammonia or urea were employed as ammonia-producing compounds almost 50% of the amine added contributed to the formation of the pyridine ring.

In addition, pyridines were also produced to a high extent in the presence of glutamine or ammonium chloride. On the contrary, the lowest amounts of pyridines were produced in the presence of creatinine.

Table 7. Pyridines produced by crotonaldehyde cyclization/oligomerization in the presence of ammonia and ammonia-producing compounds.

Ammonia-producing compound	Pyridines (µmol/mmol of amino compound)			
	2-methyl	**3-methyl**	**2,5-dimethyl**	**5-ethyl-2-methyl**
Ammonia	22.89 ± 7.01 [a]	1.10 ± 0.13 [a]	4.44 ± 0.96 [a, b]	463.3 ± 81.1 [a]
Ammonium chloride	9.56 ± 1.52 [b, c]	4.41 ± 0.98 [b]	2.04 ± 0.66 [c, d]	258.1 ± 37.4 [b]
Glutamine	14.96 ± 0.33 [c]	3.34 ± 0.66 [b]	3.66 ± 0.70 [b, d]	321.6 ± 11.9 [b, d]
Creatinine	7.59 ± 2.34 [b, c]	1.11 ± 0.31 [a]	2.09 ± 0.39 [c, d]	119.8 ± 6.9 [c]
Urea	27.51 ± 4.10 [a]	1.82 ± 0.28 [a]	5.70 ± 1.10 [a, b]	388.9 ± 59.0 [a, d]

Values are mean ± standard deviation (SD) for, at least, three independent experiments. Means in the same column with a different letter are significantly different ($p < 0.05$).

Glutamine was selected for the experiments described in the following sections because of pyridines were produced to a high extent in the presence of it and glutamine is available in many foods.

4.1.1.2. Formation of pyridines by cyclization of reactive carbonyls in the presence of glutamine

When glutamine was heated in the presence of reactive carbonyls diverse pyridines were produced (**Table 8**). The kind of pyridine and the reaction yield depended on the involved reactive carbonyl. Thus, some reactive carbonyls produced some pyridines specific with preference to others.

Table 8. Pyridines produced by reactive carbonyl cyclization in the presence of glutamine.

Pyridine	RI	Identification	Amount (µmol/mmol glutamine)	Carbonyl compound or precursor
2-Methyl	814	RI, MS, ST	9.93 ± 1.35 [a]	CROT
			34.07 ± 1.33 [b]	HxD
			13.90 ± 2.79 [a,c]	ACET
			16.58 ± 1.04 [c]	CROT/HxD
			1.55 ± 0.26 [d]	ACET/ACR
			50.21 ± 1.66 [e]	ACET/CROT
			2.46 ± 0.59 [d]	PROP/CROT
			1.59 ± 0.51 [d]	ACR/Ala
3-Methyl	863	RI, MS, ST	17.46 ± 1.47 [a]	ACR
			12.06 ± 1.40 [a,b]	HxD
			2.37 ± 0.82 [c,d]	ACET
			3.56 ± 0.46 [c,d]	PROP
			0.71 ± 0.08 [c,d]	Ala
			11.04 ± 3.81 [a,b]	ACR/CROT
			4.92 ± 1.03 [b,d]	ACR/PENT
			7.08 ± 1.05 [b,d]	ACR/HxD
			5.49 ± 1.44 [b,d]	CROT/HxD
			45.05 ± 0.82 [e]	ACET/ACR
			1.40 ± 0.04 [c,d]	ACET/CROT
			67.02 ± 4.18 [f]	PROP/ACR
			1.70 ± 0.36 [c,d]	PROP/CROT
			30.06 ± 0.65 [g]	ACR/Ala
			5.23 ± 1.79 [b,d]	BUT/ACR
2-Ethyl	909	RI, MS, ST	30.95 ± 0.34 [a]	HpD
			1.85 ± 0.33 [b]	ACR/HpD
			19.01 ± 0.50 [c]	CROT/HpD
			8.81 ± 1.54 [d]	ACE/PENT

2,5-Dimethyl	938	RI, MS, ST	8.61 ± 1.43^{a}	ACET
			0.75 ± 0.04^{b}	PROP
			5.13 ± 0.56^{c}	ACR/CROT
			3.66 ± 0.39^{d}	CROT/HpD
			1.35 ± 0.47^{b}	ACET/ACR
			7.22 ± 0.31^{a}	ACET/CROT
			82.82 ± 0.75^{e}	PROP/CROT
			0.85 ± 0.08^{b}	ACR/Ala
2,6-Dimethyl	889	RI, MS, ST	26.93 ± 1.38^{a}	HDO
			3.48 ± 0.47^{b}	MHDO
3-Ethyl	965	RI, MS, ST	17.85 ± 0.59^{a}	HpD
			$15.93 \pm 4.43^{a,b}$	ACET
			$4.89 \pm 1.69^{c,d}$	ACR/CROT
			$8.60 \pm 0.80^{c,e}$	CROT/HpD
			$5.91 \pm 0.66^{c,d}$	ACET/ACR
			1.92 ± 0.08^{d}	ACET/CROT
			2.63 ± 0.31^{d}	ACR/Ala
			$10.99 \pm 2.05^{b,e}$	BUT/ACR
2-Propyl	1003	MS	27.15 ± 1.82^{a}	OD
			15.61 ± 1.04^{b}	ACE/HEX
5-Ethyl-2-methyl	1034	RI, MS, ST	321.6 ± 11.9^{a}	CROT
			$32.63 \pm 1.51^{b,c}$	ACET
			2.55 ± 0.96^{d}	ACR/CROT
			24.33 ± 2.14^{b}	CROT/PENT
			63.77 ± 3.05^{e}	CROT/HxD
			43.58 ± 3.79^{c}	CROT/HpD
			171.5 ± 12.5^{f}	ACET/CROT
			$33.32 \pm 8.76^{b,c}$	PROP/CROT
3-Propyl	1060	MS	12.12 ± 1.38	OD

2-Butyl	1102	MS	22.42 ± 1.03	ND
2,5-Diethyl	1123	MS	19.09 ± 2.13	CROT/PENT
3-Butyl	1163	MS	12.18 ± 0.33	ND
2-Pentyl	1205	RI, MS, ST	14.68 ± 0.58	DD
3-Pentyl	1263	MS	8.42 ± 0.53	DD
2-Ethyl-5-propyl	1211	MS	101.9 ± 12.5 [a]	PENT
			1.83 ± 0.25 [b]	ACR/PENT
			29.95 ± 8.44 [c]	CROT/PENT
			1.34 ± 0.06 [b]	ACR/CROT/PENT
5-Butyl-2-propyl	1398	MS	156.9 ± 18.0 [a]	HEX
			82.24 ± 10.94 [b]	ACET/HEX
2-Butyl-5-pentyl	1599	MS	257.8 ± 9.3	HEP
5-Hexyl-2-pentyl	1806	MS	336.6 ± 43.9	OCT

Values are mean ± standard deviation (SD) for, at least, three independent experiments. For each pyridine, means in the same column with a different letter are significantly different ($p < 0.05$). Methods employed for identification: RI, retention index; MS, mass spectrum; ST, co-elution with a reference compound. Identifications carried out exclusively on the basis of MS should be considered only tentative. Abbreviations: ACET, acetaldehyde; ACR, acrolein; Ala, alanine; BUT, butyraldehyde; CROT, crotonaldehyde; DD, 2,4-decadienal; HDO, 3,5-heptadien-2-one; HEP, 2-heptenal; HEX, 2-hexenal; HpD, 2,4-heptadienal; HxD, 2,4-hexadienal; MHDO, 6-methyl-3,5-heptadien-2-one; ND, 2,4-nonadienal; OCT, 2-octenal; OD, 2,4-octadienal; PENT, 2-pentenal; PROP, propanal.

The formation of 2-alkylpyridines was promoted in the presence of 2-alkenals, acetaldehyde, and 2,4-alkadienals. Thus, the mixture of acetaldehyde and crotonaldehyde produced 50.21 ± 1.66 μmol of 2-methylpyridine per mmol

of glutamine. The amount of 2-methylpyridine produced by 2,4-hexadienal was slightly lower (34.07 ± 1.33 μmol/mmol of glutamine). Other 2,4-alkadienals also produced other 2-alkylpyridines to high extent. Thus, 2-ethylpyridine (30.95 ± 0.34 μmol/mmol of glutamine) was produced from 2,4-heptadienal, 2-propylpyridine (27.15 ± 1.82 μmol/mmol of glutamine) was produced from 2,4-octadienal, 2-butylpyridine (22.42 ± 1.03 μmol/mmol of glutamine) was produced from 2,4-nonadienal, and 2-pentylpyridine (14.68 ± 0.58 of μmol/mmol of glutamine) was produced from 2,4-decadienal. As observed, there was a decrease in the amount of pyridine produced when the chain length of the reactive carbonyl increased.

Similarly to the formation of 2-alkylpyridines, 3-alkylpyridines were also produced as a consequence of the heating of some reactive carbonyls in presence of glutamine. The formation of 3-methylpyridine was mainly promoted by acrolein and mostly in the presence of alkanals. Thus, the mixture of acrolein and propanal produced 67.02 ± 4.18 μmol/mmol of glutamine. In addition, the mixture of acrolein and acetaldehyde produced 45.05 ± 0.82 μmol/mmol of glutamine and the mixture of acrolein and alanine produced 30.06 ± 0.65 μmol/mmol of glutamine (alanine is a precursor of acetaldehyde). Acrolein alone also produced 3-methylpyridine, but to a lower extent (17.46 ± 1.47 μmol/mmol of glutamine). Finally, 2,4-hexadienal also produced 3-methylpyridine to a significant extent (12.06 ± 1.40 μmol/mmol of glutamine), although to a lower extent than 2-methylpyridine. Formation of 3-alkylpyridines by other 2,4-alkadienals was also usually observed.

Although, the formation of 2,5-dimethylpyridine was produced in the presence of only acetaldehyde or crotonaldehyde, the highest reaction yields were obtained in mixtures of propanal and crotonaldehyde. Similarly, 2,5-diethylpyridine was mostly produced in the presence of 2-pentenal and crotonaldehyde.

In addition to 2,5-diethylpyridine, in which the two substituents are identical, 2,5-dialkylpyridines with different substituents were also produced. In fact, these pyridines were produced to a high extent and in the presence of only one reactive carbonyl. This suggested that they might be produced by oligomerization of the involved reactive carbonyl. Thus, 5-ethyl-2-methylpyridine was mainly produced by oligomerization of crotonaldehyde, 2-ethyl-5-propylpyridine was mainly produced by oligomerization 2-pentenal, 5-butyl-2-propylpyridine was produced by oligomerization of 2-hexenal, 2-butyl-5-pentylpyridine was produced by oligomerization of 2-heptenal, and 5-hexyl-2-pentylpyridine was produced by oligomerization 2-octenal.

Different to other pyridines, 2,6-dialkylpyridines were not produced by oligomerization/cyclization of aldehydes. In fact, they were produced by oligomerization of ketones. Thus, 2,6-dimethylpyridine was mainly produced by heating of 3,5-heptadien-2-one. This pyridine was also produced, although to a lower extent, by heating 6-methyl-3,5-heptadien-2-one in presence of glutamine.

4.1.2. Formation of 3-hydroxypyridines as a consequence of lipid oxidation

Differently to alkylpyridines, the presence of hydroxypyridines has been less frequently described, most probably because of detection problems caused by the presence of the hydroxyl group. Nevertheless, these compounds are formed in processed foods. The hydroxyl group is mainly present at position 3 of the pyridine ring and an alkyl group can also be present at position 2.

The most common pyridine of this kind is 3-hydroxypyridine (pyridine-3-ol), which has been found in coffee (Amanpour & Selli, 2016; Moon & Shibamoto, 2009), tea (Kuo *et al.*, 2011), roasted sesame seeds (Jeong *et al.*, 2004) and the oil extracted from them (Xu-Yan *et al.*, 2012), salmon pate (Olsen *et al.*, 2006), and caramel colours (Myers & Howell, 1992), among other food products.

4.1.2.1. Formation of 3-hydroxypyridines by lipid-derived reactive carbonyls in the presence of ammonia and ammonia-producing compounds

A. Formation of 3-hydroxypyridines by cyclization of lipid-derived reactive carbonyls

In an attempt to investigate the possibility that lipid-derived reactive carbonyls were also involved in the formation of 3-hydroxypyridines, different aldehydes were heated in the presence of glutamine and the formation of 3-hydroxypyridines was studied. The reactive carbonyls screened included alkanals (propanal and hexanal), 2-alkenals (2-hexenal and 2-heptenal), 2,4-alkadienals (2,4-hexadienal, 2,4-heptadienal, 2,4-octadienal, 2,4-nonadienal, and 2,4-decadienal), 4-hydroxy-2-alkenals (4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal), 4-oxo-2-alkenals (4-oxo-2-hexenal and 4-oxo-2-nonenal), and 4,5-epoxy-2-alkenals (4,5-epoxy-2-hexenal, 4,5-epoxy-2-heptenal, and 4,5-epoxy-2-decenal). Obtained results showed that 4,5-epoxy-2-alkenals and 2,4-alkadienals were the lipid-reactive carbonyls responsible for the formation of 3-hydroxypyridines. As an example of the obtained results, **Figure 36** shows the production of 3-hydroxy-2-methylpyridine in mixtures containing glutamine and different aldehydes with the same number of carbon atoms (hexanal, 2-hexenal, 2,4-hexadienal, 4-hydroxy-2-hexenal, 4-oxo-2-hexenal, and 4,5-epoxy-2-hexenal).

As observed in the **Figure 36**, 2,4-hexadienal and 4,5-epoxy-2-hexenal produced 3-hydroxy-2-methylpyridine to a similar extent when the reaction was carried out under air. However, when the reaction was carried out under nitrogen, the amount of 3-hydroxy-2-methylpyridine produced by 2,4-hexadienal was considerably reduced. Nevertheless, the amount of 3-hydroxy-2-methylpyridine produced by 4,5-epoxy-2-hexenal remained unchanged. This suggested that 2,4-hexadienal had to be oxidized previously to be converted into 3-hydroxypyridine.

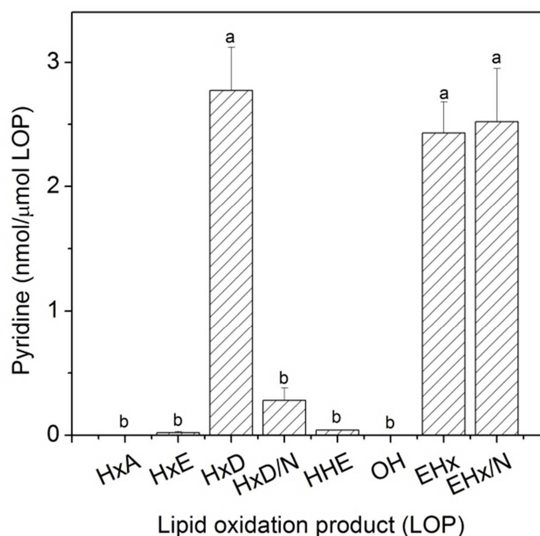


Figure 36. Effect of different lipid oxidation products on 3-hydroxy-2-methylpyridine formation. Abbreviations: HxA, hexanal; HxE, 2-hexenal; HxD, 2,4-hexadienal; HxD/N, 2,4-hexadienal under nitrogen; HHE, 4-hydroxy-2-hexenal; OHx, 4-oxo-2-hexenal; EHx, 4,5-epoxy-2-hexenal; and EHx/N, 4,5-epoxy-2-hexenal under nitrogen; LOP, lipid oxidation product. Means values with different letters are significantly different ($p < 0.05$) from three independent experiments.

Because 2,4-alkadienals and 4,5-epoxy-2-alkenals produced 3-hydroxy-2-methylpyridine similarly, the rest of the studies described in this reaction was carried out with 2,4-alkadienals because they are commercial products.

In addition to the kind of the lipid-derived reactive carbonyl, the effect of chain length on the formation of 3-hydroxypyridines was also studied and the results are shown in **Figure 37**. Five 2,4-alkadienals were studied comparatively: 2,4-Hexadienal, 2,4-heptadienal, 2,4-octadienal, 2,4-nonadienal, and 2,4-decadienal. The corresponding alkadienal with five carbons (2,4-pentadienal) could be neither obtained from commercial sources nor prepared in the laboratory. Instead of 2,4-pentadienal, 4-pentenal was employed. 4-Pentenal is also a lipid oxidation product and it has been shown to be produced as a consequence of lipid oxidation of pork meat (Park *et al.*, 2007), for example.

All assayed reactive carbonyls produced 3-hydroxypyridines that differ in the alkyl chain length present at position 2. Thus, 4-pentenal produced 3-hydroxypyridine, 2,4-hexadienal produced 3-hydroxy-2-methylpyridine, 2,4-heptadienal produced 2-ethyl-3-hydroxypyridine, and so on. As observed in the **Figure 37**, the amount of 3-hydroxypyridines produced was different for each alkadienal. The highest yield was obtained with 2,4-hexadienal and this yield decreased linearly (r^2 = 0.99, p = 0.997) as a function of the alkadienal chain length.

As observed, the amount of 3-hydroxypyridine produced by 4-pentenal was far from the regression line, which is likely a consequence of the different chemical structure of this aldehyde in relation to that of 2,4-alkadienals.

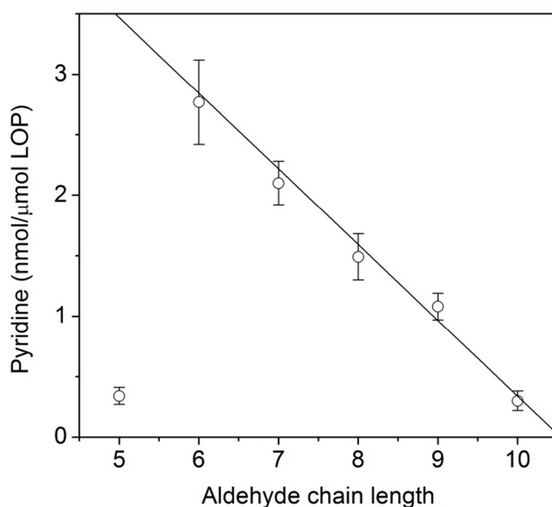


Figure 37. Effect of the chain length of the 2,4-alkadienal on the amount of 3-hydroxypyridines produced. The compounds tested were 2,4-hexadienal (6 carbon atoms), 2,4-heptadienal (7 carbon atoms), 2,4-octadienal (8 carbon atoms), 2,4-nonadienal (9 carbon atoms), and 2,4-decadienal (10 carbon atoms). The compound with 5 carbon atoms tested was 4-pentenal because the corresponding 2,4-alkadienal was not available. Abbreviation: LOP, lipid oxidation product.

B. Effect of ammonia and ammonia-producing compounds on the formation of 3-hydroxy-2-methylpyridine by cyclization of 2,4-hexadienal.

The effect of ammonia and ammonia-producing compounds on the formation of 3-hydroxy-2-methylpyridine was also studied. The obtained results are shown in **Figure 38**. As ammonia-producing compounds, ammonia, ammonium chloride, urea, glutamine, alanine, and creatinine were selected. All of them are well-known ammonia producers.

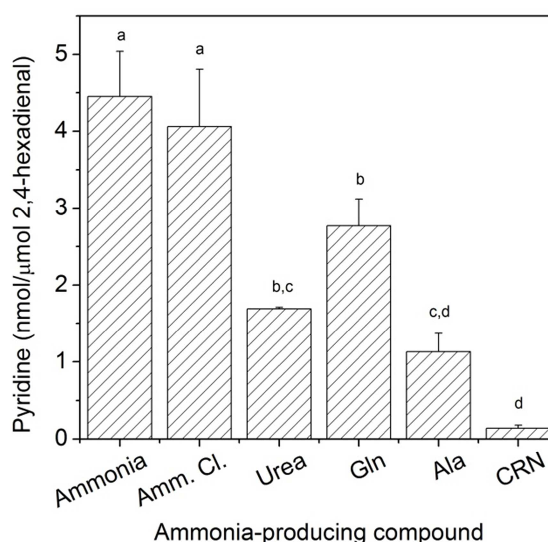


Figure 38. Effect of the type of ammonia-producing compound on 3-hydroxy-2-methylpyridine formation by cyclization of 2,4-hexadienal. Abbreviations: AmmCl, ammonium chloride; Gln, glutamine; Ala, alanine; CRN, creatinine. Means values with different letters are significantly different ($p < 0.05$).

As observed in **Figure 38**, all of them produced the formation of 3-hydroxy-2-methylpyridine. However, this pyridine was produced to different extents depending on the employed ammonia-producing compounds. The compounds that most contributed to pyridine formation were ammonia and ammonium chloride. Following these two compounds, in a decreasing order, appeared glutamine, urea, alanine, and creatinine. Because glutamine was the ammonia-producing compound employed in the study of the formation of alkylpyridines

described in section 4.1.1.1, this amino acid was also selected for the experiments carried out in this section.

C. Effect of reaction conditions on the formation of 3-hydroxy-2-methylpyridine by cyclization of 2,4-hexadienal.

This subsection describes the effect of pH, precursors, and reaction time and temperature, on the formation of 3-hydroxy-2-methylpyridine by cyclization of 2,4-hexadienal.

The amount of 3-hydroxy-2-methylpyridine produced depended on the pH of the reaction (**Figure 39**). Thus, the amount of pyridine increased as a function of reaction pH from pH 3 until pH 7, and decreased afterwards.

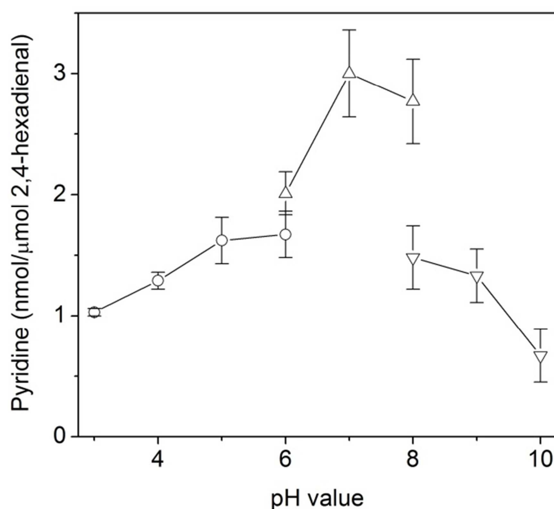


Figure 39. Effect of reaction pH on 3-hydroxy-2-methylpyridine formation by cyclization of 2,4-hexadienal in the presence of glutamine. The employed buffers were sodium citrate (○), sodium phosphate (△), and sodium borate (▽).

As observed in the **Figure 39**, the reaction was favoured in the presence of phosphate buffer in relation to borate buffer. The maximum yield was obtained at pH 7-8.

The concentration of the reaction precursors (2,4-hexadienal and glutamine) also influenced amount of the 3-hydroxy-2-methylpyridine formed. Thus, the amount of 3-hydroxy-2-methylpyridine produced increased linearly ($r^2 = 0.98$, $p < 0.0001$) between 0 and 25 μmol of 2,4-hexadienal when this aldehyde was heated in the presence of 5 μmol of glutamine (**Figure 40**). Higher amounts of aldehyde did not produce further increases in the concentration of the 3-hydroxy-2-methylpyridine.

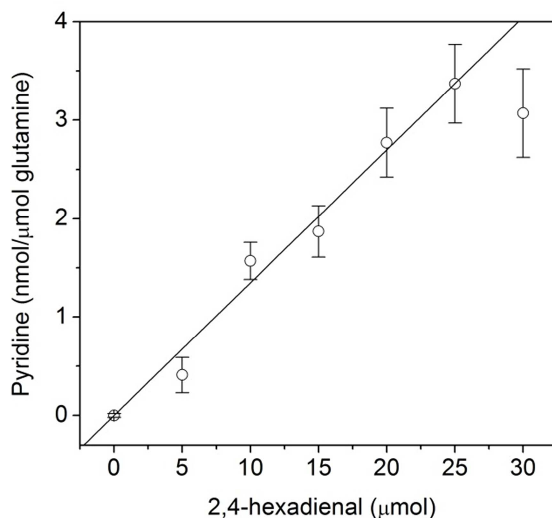


Figure 40. Effect of aldehyde concentration on 3-hydroxy-2-methylpyridine formation by cyclization of 2,4-hexadienal in the presence of glutamine.

Analogously, the amount of 3-hydroxy-2-methylpyridine produced also depended on the amount of glutamine added (**Figure 41).** Thus, in the absence of glutamine, the pyridine was not produced. However, when a small amount of glutamine was added, the 3-hydroxy-2-methylpyridine was produced to a significant extent. The highest yield for the reaction was achieved when 20 μmol of 2,4-hexadienal were heated in the presence of 5 μmol glutamine. Higher amounts of glutamine decreased the amount of 3-hydroxy-2-methylpyridine produced. Thus, when 10 μmol of glutamine was added, the amount of the produced pyridine was reduced to almost one half, and further amounts of the

amino acid continued decreasing the amount of the produced pyridine. In fact, the amount of the produced pyridine decreased linearly ($r^2 = 0.984$, $p = 0.0009$) when the amount of added glutamine increased from 10 to 30 μmol. According to the obtained results, the optimal amount of precursors for 3-hydroxy-2-methylpyridine formation were 20 μmol of 2,4-hexadienal and 5 μmol of glutamine.

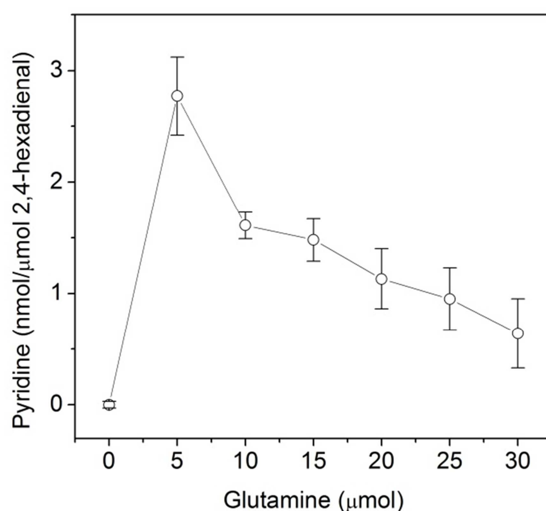


Figure 41. Effect of amino acid concentration, on 3-hydroxy-2-methylpyridine formation by cyclization of 2,4-hexadienal in the presence of glutamine.

Finally, the effect of reaction time and temperature were also studied. 3-Hydroxy-2-methylpyridine concentration increased linearly ($r^2 > 0.98$, $p < 0.005$) as a function of heating time between 100 and 180 ºC (**Figure 42**). In addition, reaction rates, which were calculated from the slopes of the obtained lines, increased as a function of the temperature. When these reaction rates were employed in an Arrhenius plot, a correlation was observed ($r^2 = 0.988$, $p = 0.0055$). From the slope of this line, an activation energy of 49.7 ± 4.5 kJ/mol was calculated for the formation of 3-hydroxy-2-methylpyridine from 2,4-hexadienal and glutamine (**Figure 43**).

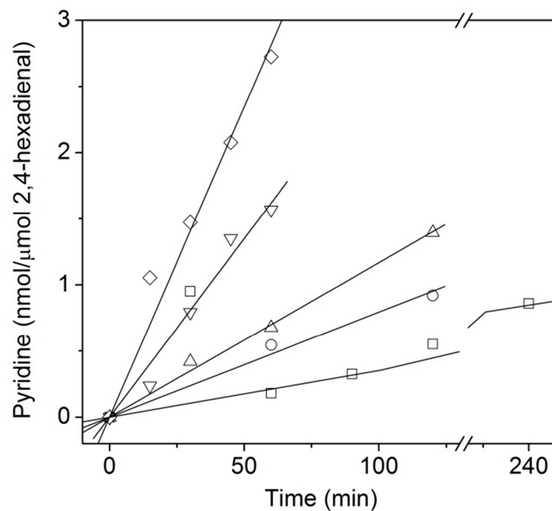


Figure 42. Effect of time and temperature on the formation of 3-hydroxy-2-methylpyridine. The temperatures assayed were: 100 (□), 120 (○), 140 (△), 160 (▽), and 180 (◇) °C.

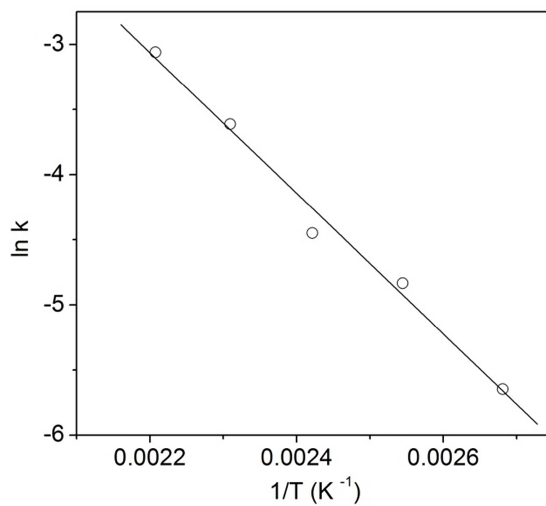


Figure 43. Arrhenius plot obtained for the formation of 3-hydroxy-2-methylpyridine.

4.1.2.2. Formation of pyridines in oxidized oils heated in the presence of glutamine.

Previous results showed that 3-hydroxypyridines were produced by lipid-derived reactive carbonyls. To confirm that lipid oxidation was involved in the formation of this type of pyridines, two polyunsaturated oils (linseed and menhaden oils) were oxidized and heated in the presence of glutamine. Both oils are known to be a good source of reactive carbonyls as a consequence of oxidation (Dlugogorski *et al.*, 2012).

Thus, the oils were oxidized under air for 5 days at 60 °C. After this time, both oils were oxidized (oxidation of the oils was verified by the appearance of hexanal in the gas chromatogram of the incubated oils, data not shown). These oxidized oils were then mixed with glutamine and heated at 100 °C for 22 h. After this time, samples were derivatizated and the amount of 3-hydroxypyridines determined. Obtained data are shown in **Table 9**.

Table 9. 3-Hydroxypyridines produced by oil oxidation in the presence of glutamine.

Oil	3-Hydroxypyridine	Amount (nmol/g of oil)
Linseed oil	3-hydroxypyridine	62 ± 11 nmol
	3-hydroxy-2-methylpyridine	34 ± 4 nmol
	2-ethyl-3-hydroxypyridine	32 ± 5 nmol
Menhaden oil	3-hydroxypyridine	117 ± 11 nmol
	3-hydroxy-2-methylpyridine	18 ± 1 nmol
	2-ethyl-3-hydroxypyridine	13 ± 2 nmol

Values are mean ± standard deviation (SD) for three independent experiments.

As can be observed, menhaden oil produced mainly 3-hydroxypyridine. Although this pyridine was also the main hydroxypyridine produced by linseed oil, this oil also produced 3-hydroxy-2-methylpyridine and 2-ethyl-3-hydroxypyridine to a significant extent. Linseed oil produced these last pyridines to a higher extent ($p < 0.05$) than menhaden oil.

4.1.3. Conversion of 2-oxofurans into 3-hydroxypyridines

Previous section has shown that 3-hydroxypyridines can be produced as a consequence of the lipid oxidation pathway. However, they can also be produced as a consequence of Maillard reaction. 2-Oxofurans are common products of Maillard reaction, in particular 5-hydroxymethylfurfural (HMF). However, when these compounds are heated in the presence of ammonia, they can suffer a ring expansion and the formation of 3-hydroxypyridines can be produced.

4.1.3.1. Conversion of HMF into 2-hydroxymethyl-5-hydroxypyridine

When HMF was heated in the presence of ammonia or ammonium chloride, the formation of 2-hydroxymethyl-5-hydroxypyridine was observed. The reaction yield depended on reaction conditions, including pH, concentrations of reactants, and time and temperature.

Figure 44 shows the effect of pH on both the formation of 2-hydroxymethyl-5-hydroxypyridine and the disappearance of HMF. As observed in **Figure 44**, 2-hydroxymethyl-5-hydroxypyridine formation was parallel to HMF disappearance. Thus, 2-hydroxymethyl-5-hydroxypyridine formation increased when the pH increased from 3 to 7, with a maximum between pH 6–8 with sodium phosphate buffer. HMF disappearance followed a similar trend. However, this trend was not observed in the presence of borate buffer. When using this buffer, the amount of produced 2-hydroxymethyl-5-hydroxypyridine was lower and an effect in relation to pH was not observed. In spite of this lower production of the hydroxypyridine, HMF was decomposed, but its decomposition did not produce the hydroxypyridine as observed in the presence of citrate or phosphate buffers. Between pH 3 and pH 8 the formation of 2-hydroxymethyl-5-hydroxypyridine and the disappearance of HMF were correlated ($r^2 = 0.9216$, $p = 0.002$). The conversion yield of the furan into the hydroxypyridine depended on the pH and ranged from 0.4% at pH 3 to 1.3% at pH 7–8.

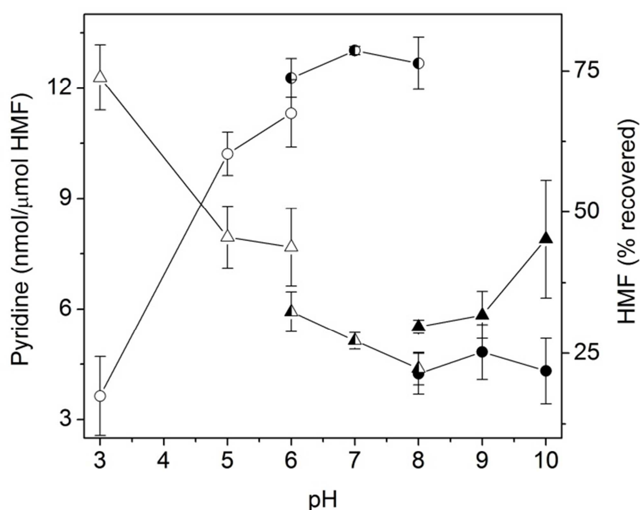


Figure 44. Effect of pH on the formation of 2-hydroxymethyl-5-hydroxypyridine (circles) and on the disappearance of 5-hydroxymethylfurfural [HMF (up pointing triangles)]. Three buffers were employed: Sodium citrate buffer (open symbols), sodium phosphate buffer (half-closed symbols), and sodium borate buffer (closed symbols).

The HMF/ammonia ratio also played a main role on the 2-hydroxymethyl-5-hydroxypyridine formation (**Figure 45**). As observed in **Figure 45,** the concentration of hydroxypyridine increased with the concentration of HMF until a maximum was achieved. Later increases in HMF concentration did not produce further increases in the amount of the produced hydroxypyridine, therefore suggesting that at high concentrations of HMF, ammonia was the limiting factor. The maximum conversion was achieved when 10 µmol of HMF were heated in the presence of 30 µmol of ammonium chloride. After that, the amount of 2-hydroxymethyl-5-hydroxypyridine remained constant. In spite of being achieved the maximum concentration of 2-hydroxymethyl-5-hydroxypyridine when 10 µmol of HMF were heated, the yield was higher when smaller amounts of HMF were employed. Thus, the yield was 3% with 2.5 µmol of HMF, and this yield decreased when higher amounts of HMF were employed.

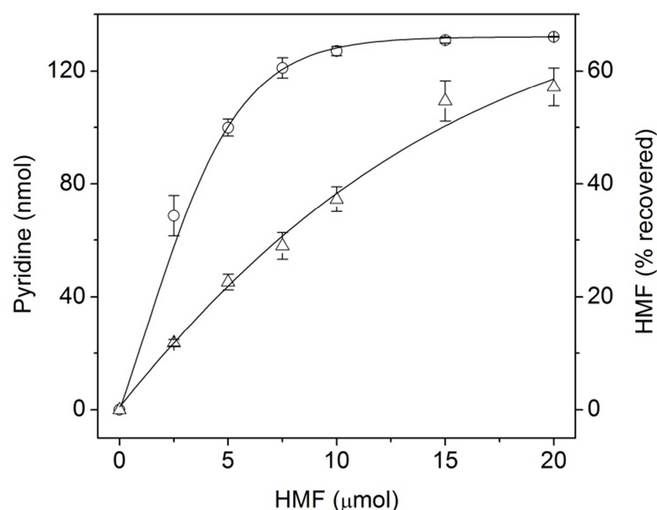


Figure 45. Effect of HMF (△) concentration on the formation of 2-hydroxymethyl-5-hydroxypyridine (○).

As expected, reaction yield also depended on the concentration of the ammonia-producing compound (**Figure 46**). Two amino compounds were assayed: Ammonia and ammonium chloride.

Both compounds exhibited a similar behaviour in relation to HMF disappearance. However, the formation of 2-hydroxymethyl-5-hydroxypyridine in the presence of ammonia chloride was slightly higher. When the amount of amino compound was added in the range 0 and 20 µmol the amount of 2-hydroxymethyl-5-hydroxypyridine produced increased linearly ($r^2 > 0.9986$, $p < 0.0007$). After that, concentration of 2-hydroxymethyl-5-hydroxypyridine produced remained constant. The inverse behaviour was observed for the HMF recovered. It decreased linearly ($r^2 = 0.992$, $p < 0.004$) between 0 and 20 µmol of the amino compound and it remained constant when more than 30 µmol of the amino compound was added. In fact, formation of 2-hydroxymethyl-5-hydroxypyridine and disappearance of HMF were correlated ($r^2 = 0.92$, $p < 0.0001$).

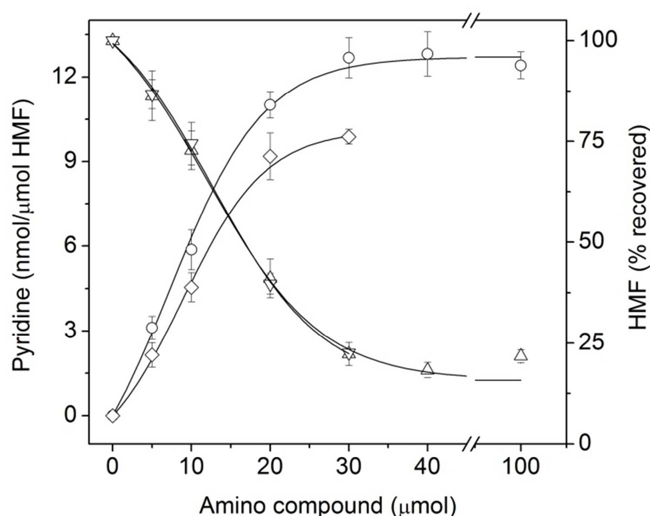


Figure 46. Effect of the concentration of the amino compound on the formation of 2-hydroxymethyl-5-hydroxypyridine (○ and ◇) and on the disappearance of HMF (△ and ▽). Two amino compounds were assayed: Ammonia (◇ and △) and ammonium chloride (○ and ▽).

HMF disappearance and 2-hydroxymethyl-5-hydroxypyridine formation also depend on time and temperature.

The time-courses of 2-hydroxymethyl-5-hydroxypyridine formation are shown in **Figure 47**. As observed in **Figure 47**, the amount of the hydroxypyridine increased linearly (r^2 = 0.686, $p < 0.04$) as a function of the heating time. Furthermore, formation rates, obtained from the slopes of the lines of the best fit, increased with temperature. In addition, a small slag period was observed. This slag was higher when temperature was lower.

Reaction rates for the formation of 2-hydroxymethyl-5-hydroxypyridine were employed in an Arrhenius plot to obtain the activation energy (**Figure 48**). The activation energy was obtained from the slope of the line of best fit and resulted to be 74 ± 3 kJ/mol.

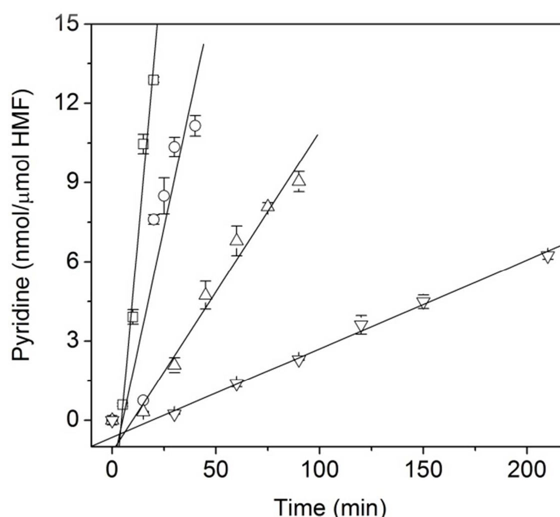


Figure 47. Effect of time and temperature on the formation of 2-hydroxymethyl-5-hydroxypyridine. The assayed temperatures were 100 (▽), 120 (△), 140 (○), and 160 (□) °C.

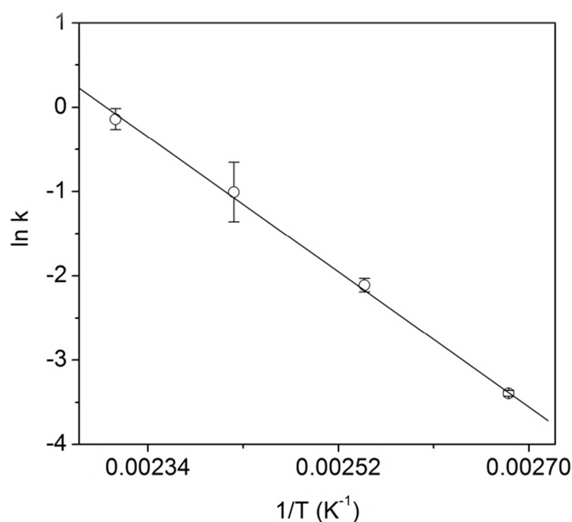


Figure 48. Arrhenius plot obtained for the formation of 2-hydroxymethyl-5-hydroxypyridine.

Similarly, **Figure 49** shows the time-courses obtained for HMF disappearance at 100–160 ºC. Recovered HMF decreased linearly ($r^2 > 0.9801$, $p < 0.04$) as a function of heating times and decomposition rates increased with temperature. In

addition, reaction rates were employed in an Arrhenius plot to obtain the activation energy for the disappearance of HMF (**Figure 50**). The activation energy was obtained from the slope of the line of best fit and resulted to be 43 ± 4 kJ/mol.

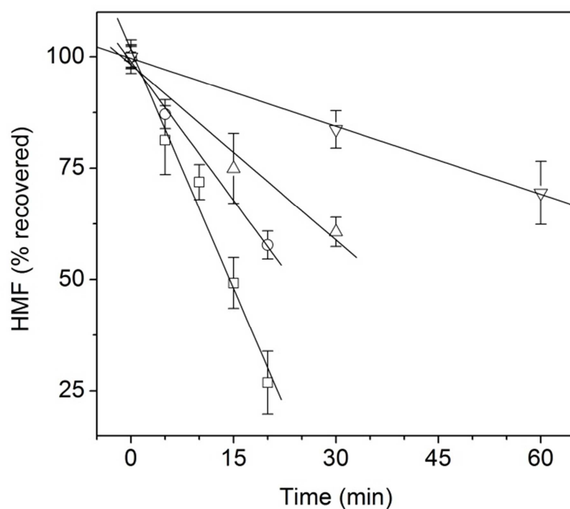


Figure 49. Effect of time and temperature on the disappearance of HMF. The assayed temperatures were 100 (▽), 120 (△), 140 (○), and 160 (□) °C.

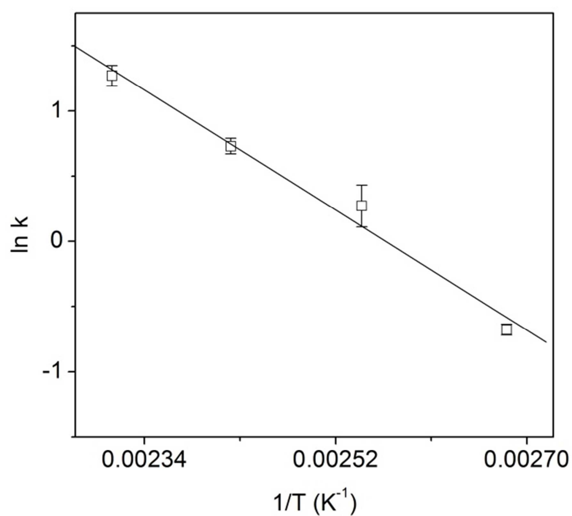


Figure 50. Arrhenius plot obtained for the disappearance of HMF.

4.1.3.2. Conversion of 2-oxofurans into 3-hydroxypyridines

In addition to HMF, other 2-oxofurans are also produced in the course of Maillard reaction. These include, for example, furfural (Lu *et al.*, 2019) and 2-acetylfuran (Kanzler *et al.*, 2017). Because of the structural similarity to that of HMF, they should be expected to be converted into the corresponding 3-hydroxypyridines. Structures for assayed furans and the produced 3-hydroxypyridines are given in **Figure 51**.

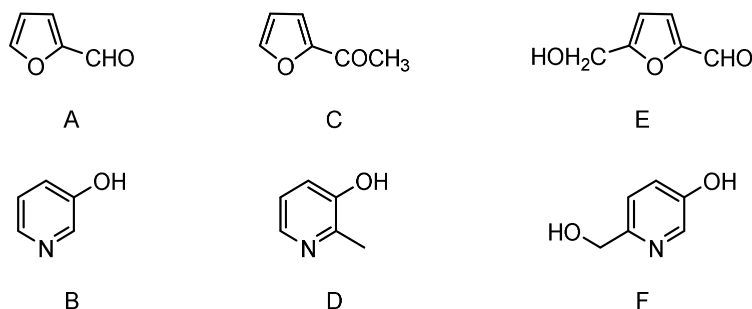


Figure 51. Structures of 2-oxofurans assayed and the corresponding 3-hydroxypyridines. A, furfural; B, 3-hydroxypyridine; C, acetylfurfural; D, 3-hydroxy-2-methylpyridine; E, HMF; F, 2-hydroxymethyl-5-hydroxypyridine.

Figure 52 shows the formation of 3-hydroxypyridines when the assayed 2-oxofurans were heated in the presence of ammonium chloride for 0, 3 and 22 hours at 100 ºC. As can be observed in **Figure 52**, the corresponding 3-hydroxypyridines were always produced. However, the conversion yield depended on the employed 2-oxofuran. Thus, the highest yield was observed for 2-hydroxymethyl-5-hydroxypyridine followed by 3-hydroxypyridine and 3-hydroxy-2-methylpyridine. 2-Hydroxymethyl-5-hydroxypyridine was produced with a yield of 0.5% after 3 h at 100 ºC, and the yield increased to 1.3% after 22 h at 100 ºC. The yield of 3-hydroxypyridine formation was 0.03% and 0.11% after 3 and 22 h, respectively. Finally, the yield for 3-hydroxy-2-methylpyridine was 0.01% and 0.06% after 3 and 22 h, respectively.

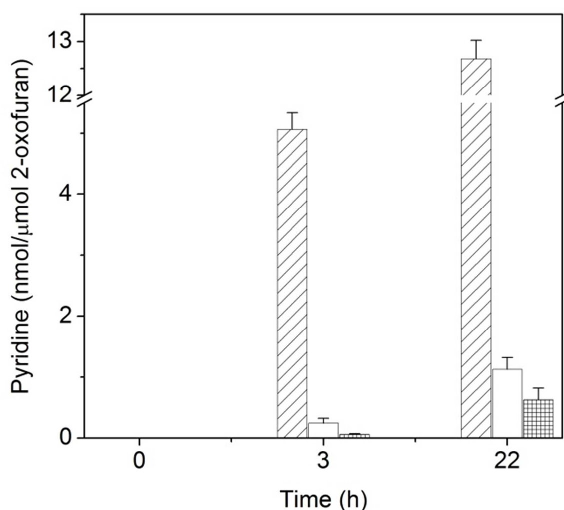


Figure 52. Formation of 3-hydroxypyridines: 2-Hydroxymethyl-5-hydroxypyridine (stripped bars), 3-hydroxypyridine (open bars), and 3-hydroxy-2-methylpyridine (crosshatched bars) from 2-oxofurans (HMF, furfural, and 2-acetylfuran, respectively).

4.1.3.3. Formation of 3-hydroxypyridines in honeys

Previous results showed that conversion of 2-oxofurans into 3-hydroxypyridines occurs easily when these compounds are heated in presence of ammonia. To confirm that this reaction can also take place in foods, a food prone to suffer Maillard reaction, such as honey, was studied.

In these studies, honeys were heated in the absence of additives. Thus, ammonia was not added because honeys contain compounds that can produce ammonia upon heating. Two different types of honey were assayed. One of them was a high quality honey, which had not suffered any previous thermal overheating. The other was a sugarcane honey, which is heated during its production process and develops Maillard reaction.

Both honeys were heated at 60 and 100 ºC and the formation of HMF and 3-hydroxypyridines was studied as a function of the heating time. **Figure 53** shows the effect of heating on the high quality honey.

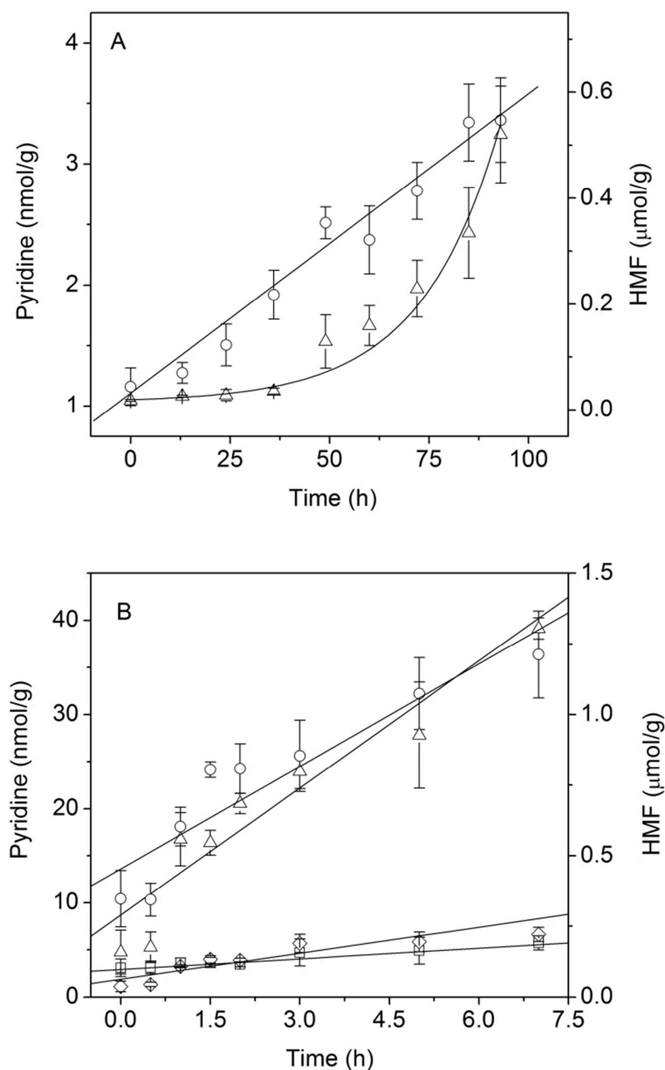


Figure 53. Effect of heating time on the formation of 2-hydroxymethyl-5-hydroxypyridine (○) and HMF (△) in honey heated at A, 60, and B, 100 °C. Honey samples (1 g) were heated in closed test tubes for the indicated times and temperatures.

As expected, very small amounts of HMF and 2-hydroxymethyl-5-hydroxypyridine were detected in this honey at the initial step, due to the absence of heating during its production. However, when the honey was heated at 100ºC, HMF and 2-hydroxymethyl-5-hydroxypyridine were produced to a significant extent. In addition, trace amounts of 3-hydroxy-2-methylpyridine were also detected. On the other hand, formation of 3-hydroxypyridine was not observed.

Significant differences were observed between the formation kinetics of HMF and 2-hydroxymethyl-5-hydroxypyridine. Thus, HMF increased exponentially as a function of heating time, as observed previously (Chua, 2018). However, 2-hydroxymethyl-5-hydroxypyridine concentration increased linearly ($r^2 = 0.8836$, $p < 0.0002$).

Results were different when sugarcane honey was studied (**Figure 54**). As expected because of the treatment at which this honey had been submitted during its production processing, 2-oxofurans and 3-hydroxypyridines were already present in the commercial honey. In addition, 2-oxofurans and 3-hydroxypyridines concentrations increased linearly ($r^2 > 0.8649$, $p < 0.0008$) upon heating. In fact, there was a correlation between them at both 60 ($r^2 > 0.9409$, $p = 0.005$) and 100 ºC ($r^2 > 0.9409$, $p < 0.0001$). Surprisingly, 2-hydroxymethyl-5-hydroxypyridine/HMF ratio was higher at 60 ºC (~0.27) than at 100 ºC (~0.034).

In addition to 2-hydroxymethyl-5-hydroxypyridine, small amounts of 3-hydroxypyridine and 3-hydroxy-2-methylpyridine were also present in sugarcane honey at the initial step and their concentrations increased linearly ($r^2 > 0.81$, $p < 0.002$) as a function of heating time and temperature. However, and analogously to the observed in the model systems and the honey, the concentrations of both pyridines were always lower than that of 2-hydroxymethyl-5-hydroxypyridine.

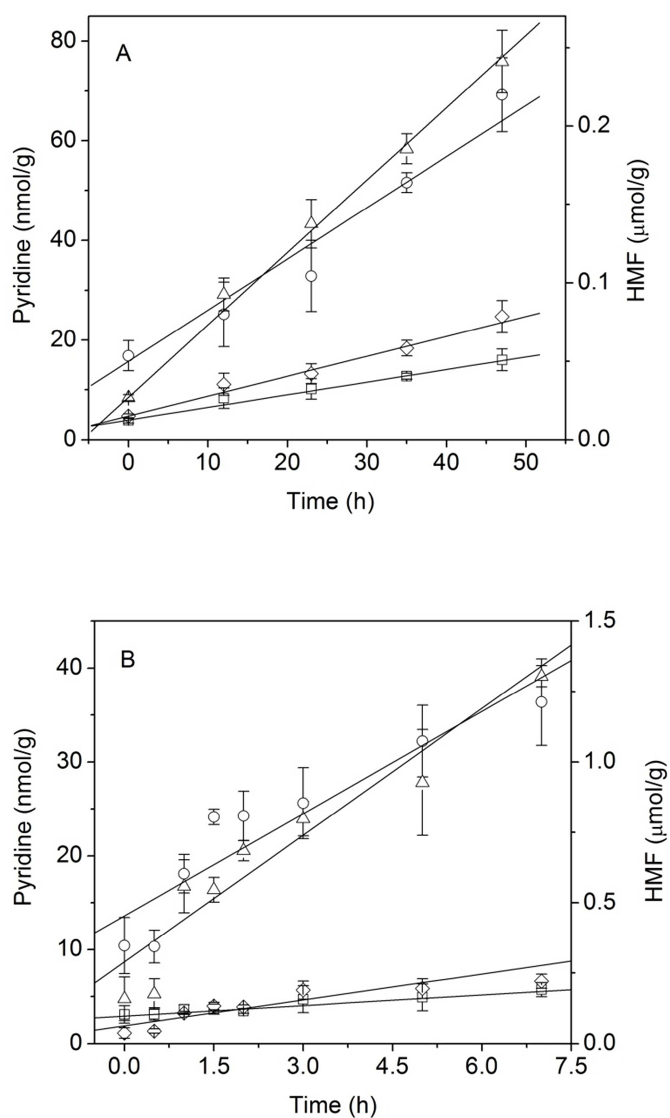


Figure 54. Effect of heating time on the formation of 2-hydroxymethyl-5-hydroxypyridine (○), HMF (△), 3-hydroxypyridine (□), and 3-hydroxy-2-methylpyridine (◇) in sugarcane honey heated at A, 60, and B, 100 °C. Sugarcane honey (1 g) was heated in closed test tubes for the indicated times and temperatures.

4.2. Cyclization and oligomerization of the reactive carbonyls in presence of creatinine: Formation of heterocyclic aromatic amines

Section 4.1 has shown that reactive carbonyls are prone to suffer cyclizations and oligomerizations to produce heterocyclic derivatives, in particular pyridines, when heated in the presence of ammonia and ammonia-producing compounds. However, if this heating is produced in the presence of other nucleophiles, these compounds might also take part in the reaction and the formation of alternative compounds might be produced.

As an example, when 2,4-hexadienal was heated in the presence of creatinine a much lower amount of 3-hydroxy-2-methylpyridine was obtained than when ammonia was employed (**Figure 38**). This might be a consequence of a low production of ammonia from creatinine, but also as a consequence of reaction between creatinine and the aldehyde. This would modify the aldehyde cyclizations described in section 4.1 and other products might be produced. This section describes the cyclization reactions of reactive carbonyls in the presence of creatinine. The formation of heterocyclic aromatic amines with the structure of aminoimidazoazarene is produced.

4.2.1. Reactive carbonyls and the formation of MeIQ

This subsection is dedicated to the identification of precursors and reaction conditions that promote the formation of MeIQ.

4.2.1.1. Formation of MeIQ in reaction mixtures of reactive carbonyls, creatinine, and amino compounds

When most reactive carbonyls were heated in the presence of creatinine and glutamine the formation of MeIQ was not detected in most assayed reaction mixtures (**Table 10**). However, when crotonaldehyde was present in the reaction mixtures, the formation of MeIQ was observed.

Table 10. MeIQ produced in mixtures of reactive carbonyls and amino compounds.

Reactions of RCO with glutamine		**Reactions of Crot with amino compounds**	
Reactive carbonyl	**MeIQ (nmol/mmol of RCO)**	**Amino compound**	**MeIQ (nmol/mmol of Crot)**
Carbohydrates	n.d. [a]	None	37 ± 7 [a,b,c]
Fatty acids and esters	n.d. [a]	Alanine	64 ± 7 [c,d]
Lipid hydroperoxides	n.d. [a]	Valine	34 ± 6 [a,b,c]
Formaldehyde	n.d. [a]	Leucine	35 ± 6 [a,b,c]
Acetaldehyde	n.d. [a]	Isoleucine	40 ± 9 [a,b,c]
Propanal	n.d. [a]	Proline	n.d. [a]
Hexanal	n.d. [a]	Phenylalanine	24 ± 10 [a,b,c]
Acrolein	n.d. [a]	Tryptophan	30 ± 5 [a,b,c]
Crotonaldehyde	202 ± 22 [b]	Methionine	48 ± 7 [b,c]
2-Pentenal	n.d. [a]	Glycine	40 ± 8 [a,b,c]
2-Octenal	n.d. [a]	Serine	109 ± 17 [e]
2,4-Heptadienal	n.d. [a]	Threonine	57 ± 6 [b,c,d]
2,4-Octadienal	n.d. [a]	Cysteine	99 ± 12 [d,e]
2,4-Decadienal	n.d. [a]	Tyrosine	41 ± 9 [a,b,c]
4-Hydroxy-2-hexenal	n.d. [a]	Asparagine	123 ± 20 [e]
4-Hydroxy-2-nonenal	n.d. [a]	Aspartic acid	99 ± 18 [d,e]
4-Oxo-2-hexenal	n.d. [a]	Glutamine	202 ± 22 [f]
4-Oxo-2-nonenal	n.d. [a]	Glutamic acid	108 ± 12 [e]
4,5-Epoxy-2-heptenal	n.d. [a]	Lysine	25 ± 8 [a,b,c]
4,5-Epoxy-2-decenal	n.d. [a]	Arginine	45 ± 2 [b,c]
Glyoxal	n.d. [a]	Histidine	46 ± 5 [b,c]
Malondialdehyde	n.d. [a]	Allylamine	18 ± 4 [a,b]
Fumaraldehyde	n.d. [a]	Ammonia	191 ± 28 [f]
Furfural	n.d. [a]	Ammonium chloride	195 ± 33 [f]
Acet/Crot (1:1)[$]	192 ± 21 [b]		
Acet/Crot (2:1)[$]	140 ± 33 [c]		

Means in the same column with different letter are significantly ($p < 0.05$) different. Abbreviations: Acet, acetaldehyde; Crot, crotonaldehyde; RCO, reactive carbonyl; n.d., not detected. $ Data are given in nmol/mmol of crotonaldehyde.

Furthermore, when the same amount of acetaldehyde and crotonaldehyde were heated in the presence of creatinine and glutamine, the amount of MeIQ produced was not significantly different ($p < 0.05$) to that produced in absence of acetaldehyde. However, in the presence of a double amount of acetaldehyde, the formation of MeIQ was reduced.

Table 10 also shows the MeIQ produced in mixtures of reactive carbonyls and amino compounds. Differently to the observed with reactive carbonyls, when different amino compounds were assayed, all of them produced MeIQ, including the control (only creatinine was added). However, significant ($p < 0.05$) differences among the assayed amino compounds were observed. The compounds that produced MeIQ to the highest extent were glutamine, ammonia, and ammonium chloride. In addition, the following compounds also produced more MeIQ than the control: Asparagine, serine, glutamic acid, aspartic acid, and cysteine. Because glutamine was used previously to induce cyclization of reactive carbonyls, this compound was also selected for studying the formation of heterocyclic aromatic amines.

4.2.1.2. Effect of reaction conditions on the formation of MeIQ in reaction mixtures of crotonaldehyde, creatinine, and glutamine

Once identified the precursor of MeIQ, the effect of the concentration of the reactants and the reaction conditions on MeIQ formation was studied. The studied parameters were pH, reactant concentrations (amount of crotonaldehyde, creatinine and glutamine), water content, presence of oxygen, and time and temperature.

The effect of reaction pH on MeIQ formation is shown in **Figure 55.** MeIQ was mainly produced at almost neutral pH and in the presence of phosphate buffer. When pH increased, a lineal decrease in the amount of MeIQ produced between pH 6.5 and pH 9 was observed ($r^2 = 0.994$, $p = 0.003$). The maximum

amount of MeIQ was obtained at pH close to 6.5. This was the pH selected for the other experiments.

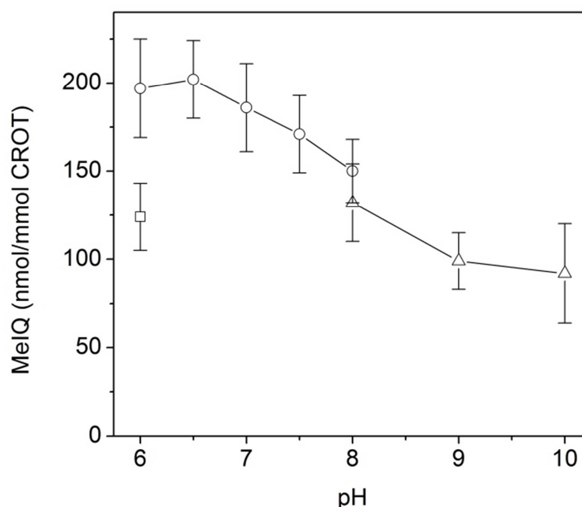


Figure 55. Effect of reaction pH on MeIQ formation by reaction of crotonaldehyde, creatinine, and glutamine. The employed buffers were sodium citrate (□), sodium phosphate (○), and sodium borate (△).

MeIQ formation also depended on the concentration of the reactants. As shown in **Figure 56,** MeIQ was not produced in the absence of crotonaldehyde. However, even small amounts of crotonaldehyde induced MeIQ formation. In fact, the amount of MeIQ produced increased linearly ($r^2 = 0.974$, $p < 0.0001$) as a function of the amount of crotonaldehyde added in the range 0–80 µmol (**Figure 56**). Additional increases of crotonaldehyde concentration produced the decrease of the MeIQ formed.

A similar behaviour was observed when different amounts of creatinine were added (**Figure 57**). In the absence of creatinine, MeIQ was not detected. In addition, MeIQ concentration increased linearly ($r^2 = 0.996$, $p < 0.0001$) as function of creatinine concentration in the range 0–20 µmol.

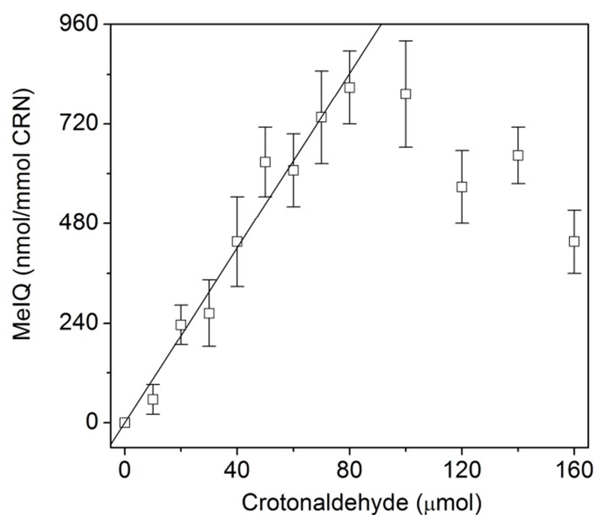


Figure 56. Effect of the concentration of crotonaldehyde on the MeIQ produced by reaction of crotonaldehyde, creatinine, and glutamine. Abbreviation: CRN, creatinine.

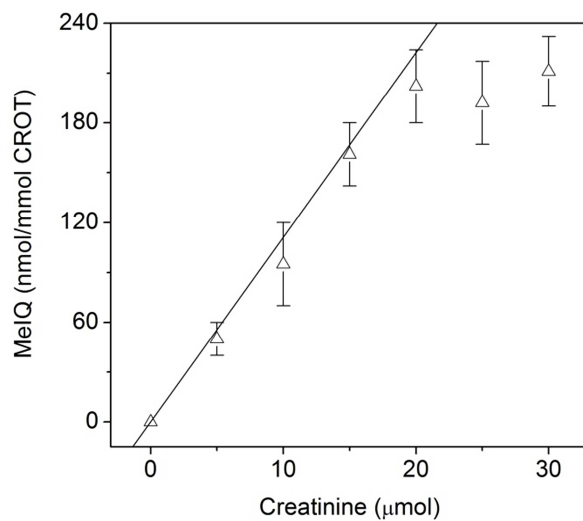


Figure 57. Effect of the concentration of creatinine on the MeIQ produced by reaction of crotonaldehyde, creatinine, and glutamine. Abbreviation: CROT, crotonaldehyde.

The maximum amount of MeIQ obtained was produced when 20 µmol of creatinine were heated in the presence of 80 µmol of crotonaldehyde. Additional amounts of creatinine did not increase the amount of produced MeIQ. However, differently to that observed with the addition of crotonaldehyde, the amount of produced MeIQ did not decrease.

Differently to crotonaldehyde and creatinine, MeIQ was produced in the absence of glutamine. In addition, only small amounts of glutamine were needed to achieve the maximum of MeIQ and additional amounts of glutamine decreased the MeIQ produced (**Figure 58**). In fact, the maximum amount of MeIQ was produced around 12 µmol.

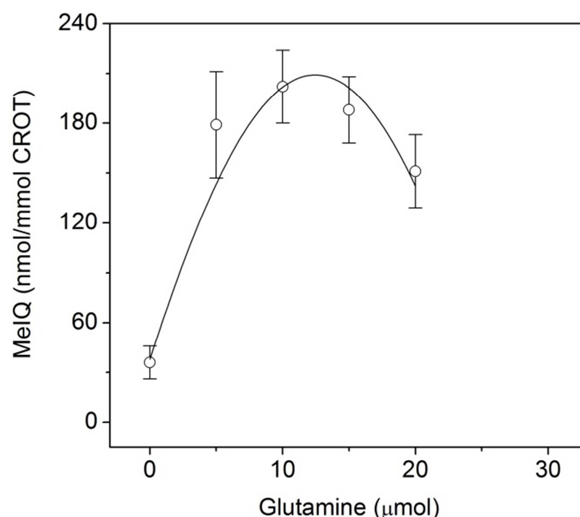


Figure 58. Effect of the concentration of glutamine on the MeIQ produced by reaction of crotonaldehyde, creatinine, and glutamine. Abbreviation: CROT, crotonaldehyde.

MeIQ formation also depended on the amount of water added to the system (**Figure 59**). The maximum concentration of MeIQ was produced when 35–55 µL of water were added, and the MeIQ formed rapidly decreased when higher amounts of water were added.

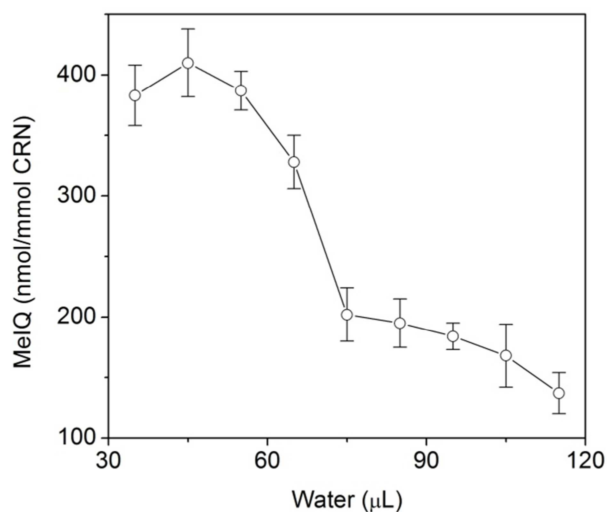


Figure 59. Effect of water concentration on MeIQ formation by reaction of crotonaldehyde, creatinine, and glutamine.

The amount of MeIQ produced also depended on the presence of oxygen (**Figure 60**).

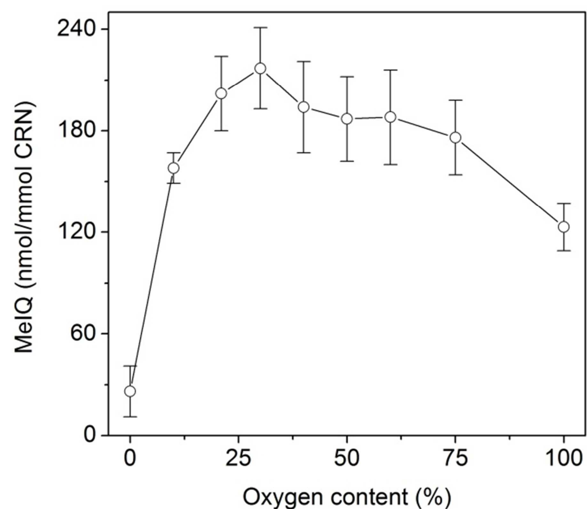


Figure 60. Effect of oxygen content on MeIQ formation by reaction of crotonaldehyde, creatinine, and glutamine.

Figure 60 shows that, in absence of oxygen, the amount of produced MeIQ was considerably reduced. In fact, it increased in the presence of oxygen. Thus, a maximum was achieved in the presence of 30% oxygen. On the other hand, higher oxygen concentrations reduced MeIQ formation.

MeIQ production also increased as function of the reaction time and temperature. **Figure 61** shows that the amount of MeIQ produced increased linearly ($r^2 > 0.98$, $p < 0.0001$) in the range 140-200 ºC. In addition, reactions rates, which were calculated from the slopes of the obtained lines, increased as a function of the temperature. When these reaction rates were employed in an Arrhenius plot (**Figure 62**), a linear regression ($r^2 = 0.99994$, $p < 0.0001$) was obtained. From the slope of this line, the activation energy of MeIQ formation was obtained. It resulted to be 72.2 ± 0.4 $kJ \cdot mol^{-1}$. This activation energy was slightly lower than that described for PhIP formation in the presence of reactive carbonyls (80.9 $kJ \cdot mol^{-1}$) (Zamora *et al.*, 2013).

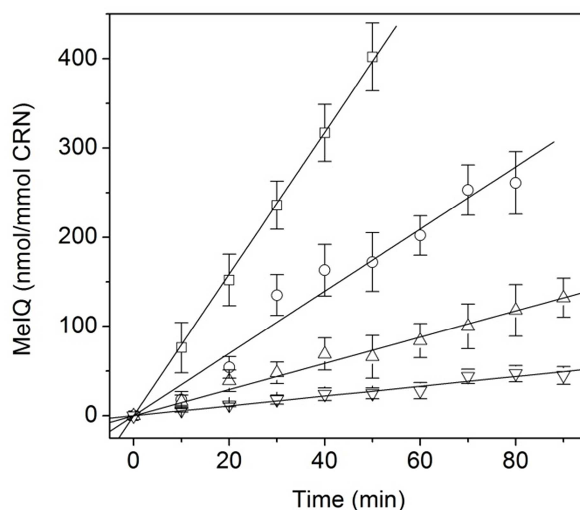


Figure 61. Effect of time and temperature on MeIQ formation by reaction of crotonaldehyde, creatinine, and glutamine. Temperatures assayed were: 140 (▽), 160 (△), 180 (○), and 200 (□) °C.

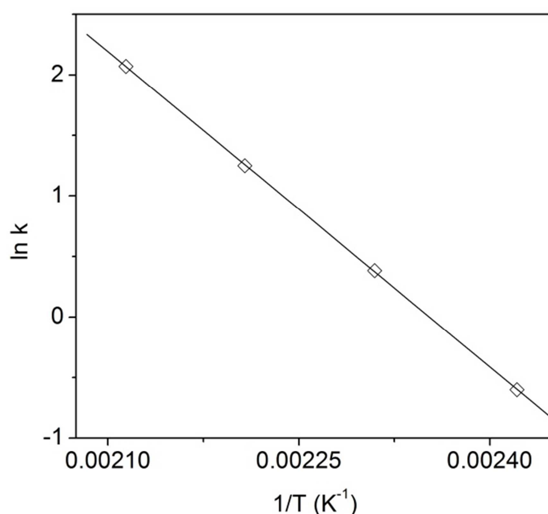


Figure 62. Arrhenius plot obtained for the formation of MeIQ.

4.2.2. Reactive carbonyls and the formation of IQ

IQ only differs from MeIQ in the methyl group at position 4 of the imidazo(4,5-*f*)quinoline structure. Therefore, and analogously to the results obtained in section 4.2.1, a role of reactive carbonyls in IQ formation should be expected.

4.2.2.1. Formation of IQ and MeIQ in mixtures of acrolein, crotonaldehyde, creatinine, and glutamine

Analogously to the above described experiments carried out to study MeIQ formation, production of IQ was screened in mixtures of carbonyl compounds derived from both carbohydrates and oxidized lipids with creatinine and glutamine. The studied carbonyl compounds included saturated, mono- and di-carbonyl compounds, 2-alkenals, 2,4-alkadienals, 4-hydroxy-2-alkenals, 4-oxo-2-alkenals, and 4,5-epoxy-2-alkenals, among others. Obtained results showed that IQ was only produced in samples involving acrolein and crotonaldehyde. In addition, the formation of MeIQ was always observed in these reaction mixtures.

As described in section 4.2.1, when reaction mixtures only included crotonaldehyde, the formation of MeIQ, but not IQ, was observed.

Reaction yield depend on the way in which the reaction was carried out. When acrolein, crotonaldehyde, creatinine, and glutamine were heated at 180ºC for 1h, only small amounts of IQ were obtained. However, when acrolein was incubated with creatinine at a low temperature for a certain time period, and then crotonaldehyde and glutamine added and heating at high temperature, the amount of produced IQ increased considerably.

Two temperatures (4 °C and 25 °C) were tested for this pre-treatment. Both temperatures produced similar results. **Figure 63** shows the results at 25 ºC.

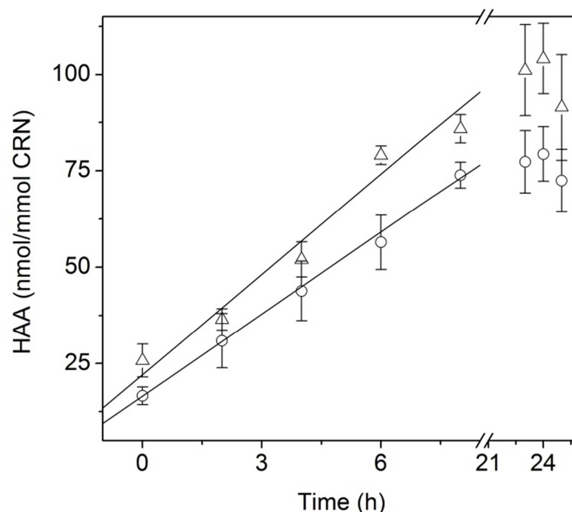


Figure 63. Effect of the reaction time of the first incubation (at 25 °C) on the formation of IQ (○) and MeIQ (△). Abbreviations: CRN, creatinine; HAA, heterocyclic aromatic amine.

As can be observed, the amounts of both IQ and MeIQ increased linearly ($r^2 > 0.96$, $p < 0.003$) as a function of incubation time for the first 8 h and, then, remained unchanged. These results suggested that this first incubation time played a major role on the amount of the heterocyclic aromatic amines obtained,

but the IQ/MeIQ ratio (≅ 0.78) was independent of this incubation time. In order to obtain high amounts of IQ and MeIQ, acrolein/creatinine samples were always pre-incubated overnight at 25 ºC before adding the crotonaldehyde and the glutamine, and heating at high temperature for a short time period.

4.2.2.2. Effect of reaction conditions on the formation of IQ and MeIQ in mixtures of acrolein, crotonaldehyde, creatinine, and glutamine

Analogously to the above described for MeIQ, the formation of IQ depend on reaction conditions, including pH, concentrations of precursors, presence of water, and reaction time and temperature.

IQ formation depended on reaction pH (**Figure 64**).

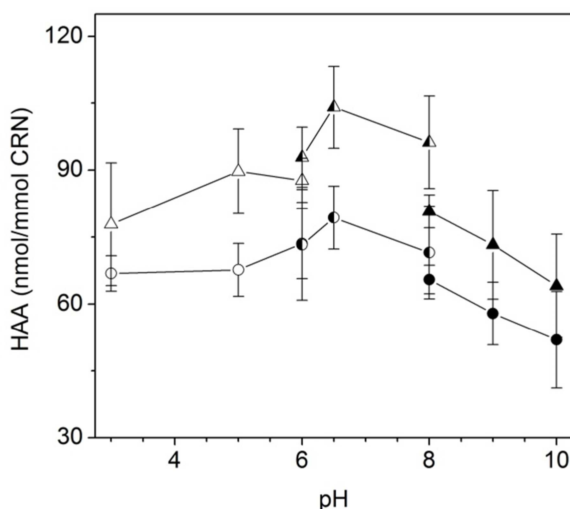


Figure 64. Effect of reaction pH on the formation of IQ (○) and MeIQ (△). Three buffers were employed: Sodium citrate (open symbols), sodium phosphate (semi-closed symbols), and sodium borate (closed symbols). Abbreviations: CRN, creatinine; HAA, heterocyclic aromatic amine.

This pH dependence was analogous to that of MeIQ. In fact, both formations were correlated ($r^2 = 0.88$, $p = 0.00014$). Thus, the amount of both IQ and MeIQ increased as a function of pH between pH 3 and pH 6.5. After achieving a

maximum, the amount of IQ and MeIQ decreased linearly ($r^2 < 0.774$, $p < 0.018$) as a function of pH. As observed in **Figure 64**, IQ and MeIQ were produced over a wide pH range, with a maximum at about pH 6.5.

IQ and MeIQ formation also depended on the concentrations of the reactants. **Figure 65** shows that increasing amounts of acrolein produced increasing amounts of IQ until achieving a maximum when 20 µmol of acrolein was added in the presence of 10 µmol of crotonaldehyde. Higher amounts of acrolein produced a decrease in the amount of IQ formed. As expected, MeIQ was produced to the highest extent when acrolein was absent and addition of acrolein always decreased the amount of MeIQ produced. The selected conditions in this study were 20 µmol of acrolein and 10 µmol of crotonaldehyde.

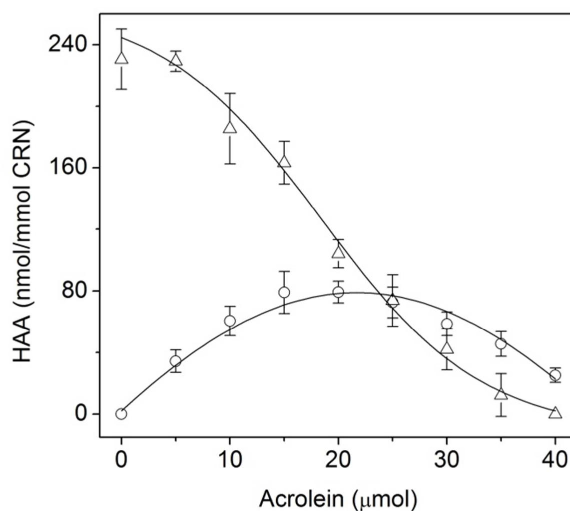


Figure 65. Effect of acrolein concentration on the formation of IQ (○) and MeIQ (△). Abbreviations: CRN, creatinine; HAA, heterocyclic aromatic amine.

IQ and MeIQ formation also depended on the concentration of creatinine (**Figure 66**).

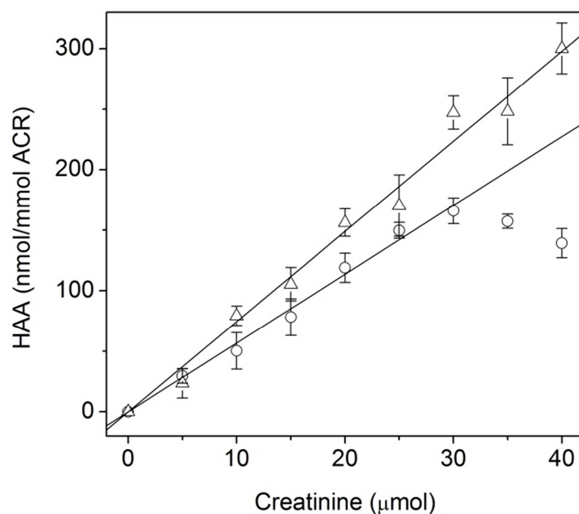


Figure 66. Effect of creatinine concentration on the formation of IQ (○) and MeIQ (△). Abbreviations: ACR, acrolein; HAA, heterocyclic aromatic amine.

In absence of creatinine, neither IQ nor MeIQ were produced. However, when the concentration of CRN increased, the concentration of both heterocyclic aromatic amines increased linearly (r^2 = 0.98, p < 0.0001) as a function of concentration creatinine in the range 0–30 μmol for IQ and 0–40 μmol for MeIQ.

IQ and MeIQ formation also depended on the presence of water (**Figure 67**). Both trends followed a Gaussian behaviour with a maximum at about 40 μL of water for IQ and 30 μL of water for MeIQ. In addition, IQ was produced to a lower extent than MeIQ when lower amount of water were added. Nevertheless, the amount of MeIQ produced decreased rapidly in the presence of high amounts of water. The amount of produced IQ and MeIQ were very similar when more than 60 μL of water was added.

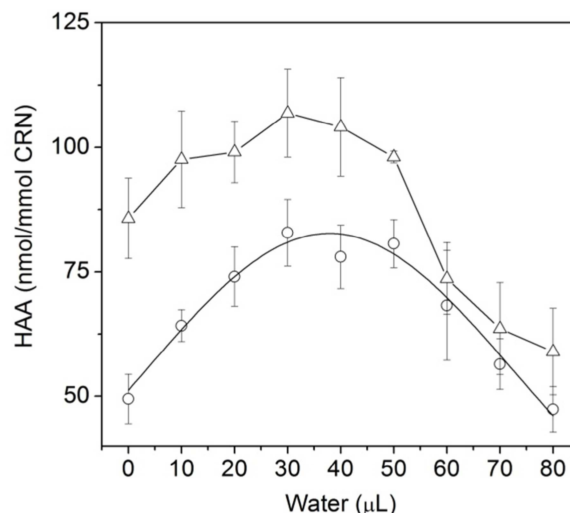


Figure 67. Effect of water added on the formation of IQ (○) and MeIQ (△). Abbreviations: CRN, creatinine; HAA, heterocyclic aromatic amine.

Time and temperature also played major roles in the formation both IQ and MeIQ. Thus, IQ formation increased linearly ($r^2 > 0.90$, $p < 0.0002$) as a function of incubation time between 140 and 200 °C (**Figure 68**).

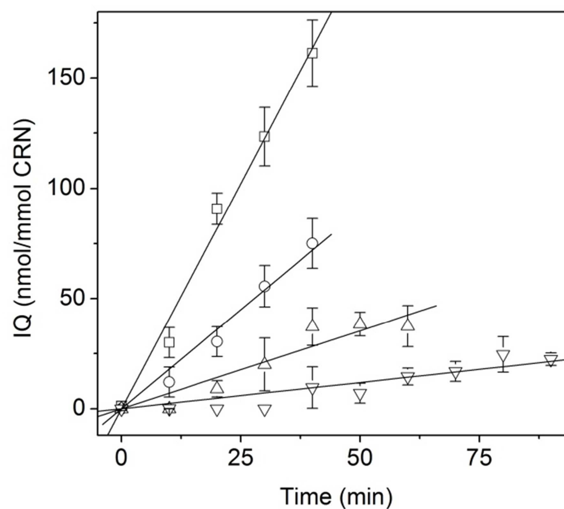


Figure 68. Time-course of IQ formation at different temperatures. Temperatures assayed were: 140 (▽), 160 (△), 180 (○), and 200 (□) °C. Abbreviation: CRN, creatinine.

A similar behaviour was observed for MeIQ formation, MeIQ also increased linearly ($r^2 > 0.90$, $p < 0.004$) as a function of incubation time (**Figure 69**).

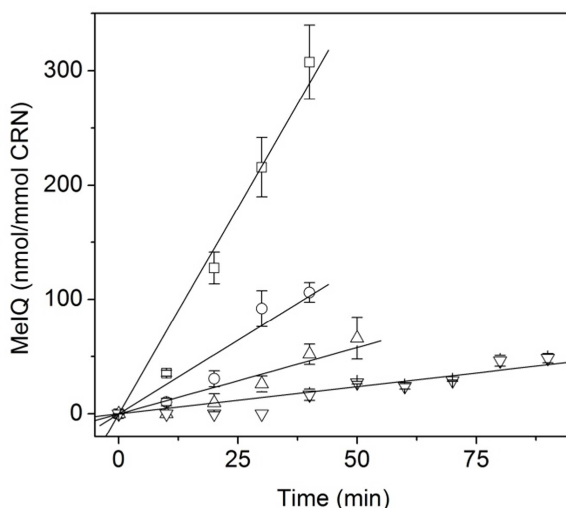


Figure 69. Time-course of MeIQ formation at different temperatures. Temperatures assayed were: 140 (▽), 160 (△), 180 (○), and 200 (□) °C. Abbreviation: CRN, creatinine.

Formation rates for both IQ and MeIQ, obtained from the slopes of the obtained lines in **Figure 68** and **Figure 69**, increased with temperature. When these formation rates were plotted against the inverse of the temperature in an Arrhenius plot, linear correlations were obtained for IQ ($r^2 = 0.9994$, $p = 0.0003$) and MeIQ ($r^2 = 0.996$, $p = 0.0023$) (**Figure 70** and **Figure 71**, respectively). From the slope of the obtained lines, the activation energies of IQ and MeIQ formation were obtained. Activation energy for IQ was 77.0 ± 1.3 kJ/mol and activation energy for MeIQ was 72.6 ± 4.3 kJ/mol. This last value was not significantly different to the value of 72.2 ± 0.4 kJ/mol obtained for MeIQ formation in the absence of acrolein as described in section 4.2.1.

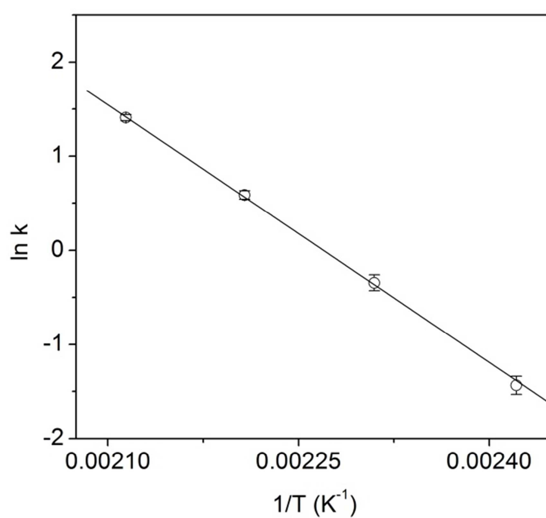


Figure 70. Arrhenius plot obtained for the formation of IQ.

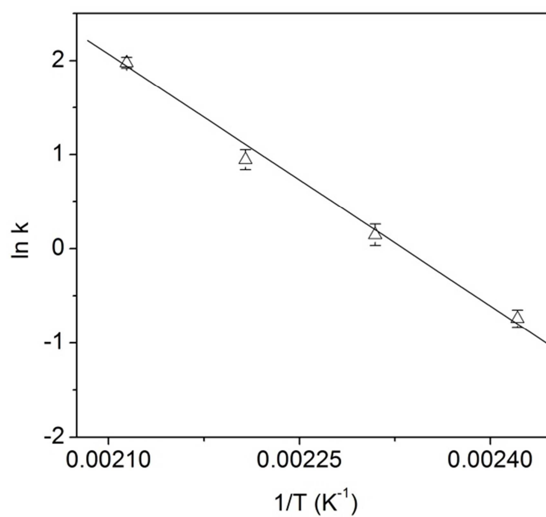


Figure 71. Arrhenius plot obtained for the formation of MeIQ.

4.2.3. Reactive carbonyls and the formation of MeIQx

Analogously to the aminoimidazoazarenes with the structure of quinoline (IQ and MeIQ), the aminoimidazoazarenes with the structure of quinoxaline can also be hypothesized to be produced by cyclization of reactive carbonyls in presence of creatinine and ammonia-producing compounds. As representative aminoimidazoazarene with the structure of quinoxaline, the formation of MeIQx was studied.

4.2.3.1. Formation of MeIQx in reaction mixtures of reactive carbonyls and creatinine

Analogously to the experiments described in previous subsections, different reactive carbonyls were screened for the formation of MeIQx. Tested carbonyls included alkanals, 2-alkenals, 2,4-alkadienals, 4-hydroxy-2-alkenals, 4-oxo-2-alkenals, 4,5-epoxy-2-alkenals, and dialdehydes. Obtained results showed that MeIQx was only observed when the assayed reactive carbonyl compound was acrolein.

Analogously to the other studied heterocyclic aromatic amines, MeIQx formation depended on reaction conditions, including pH, amount of reactants, water content, and time and temperature.

Figure 72 shows the effect of reaction pH on the formation of MeIQx. This heterocyclic aromatic amine was produced within a wide pH range, with a maximum at about pH 6.5.

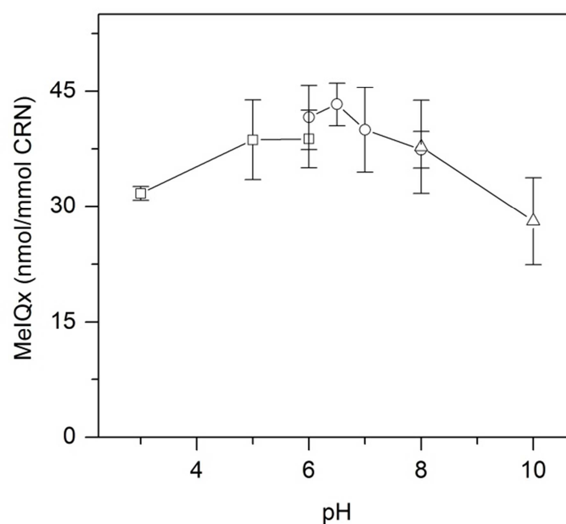


Figure 72. Effect of pH on MeIQx formation. The employed buffers were sodium citrate (□), sodium phosphate (○), and sodium borate (△).

MeIQx formation also depended on the concentration of acrolein and creatinine (**Figure 73** and **Figure 74**, respectively).

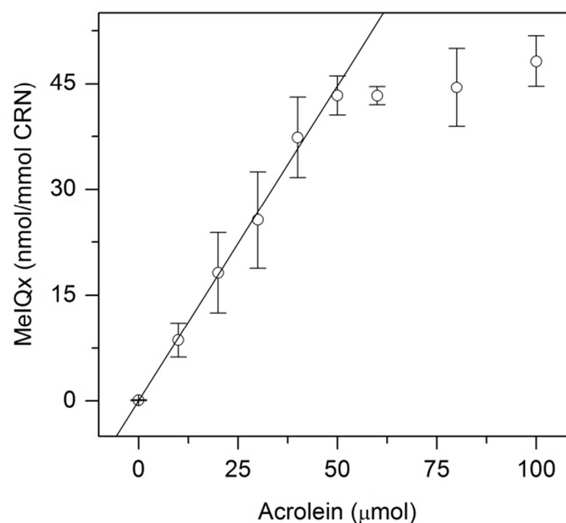


Figure 73. Effect of acrolein concentration on MeIQx formation. Abbreviation: CRN, creatinine.

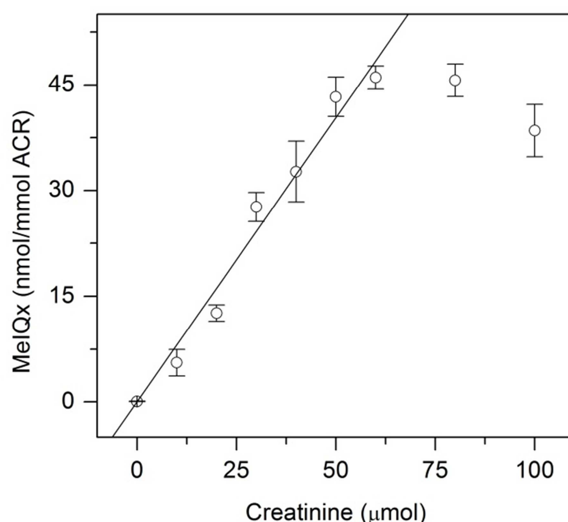


Figure 74. Effect of creatinine concentration on MeIQx formation. Abbreviation: ACR, acrolein.

Thus, when increasing amounts of acrolein were heated in presence of creatinine (50 μmol), the amount of produced MeIQx increased linearly (r^2 = 0.99, $p < 0.0001$) in the range 0–50 μmol of acrolein (**Figure 73**). Analogously, the amount of produced MeIQx also increased linearly (r^2 = 0.972, p = 0.0001) when increasing amounts of creatinine in the range 0–50 μmol were heated in the presence of 50 μmol of acrolein (**Figure 74**).

In both cases, MeIQx concentration did not increase when higher amounts of one reactant were heated in presence of 50 μmol of the other reactant. These results, suggested that acrolein and creatinine were the main reactants for MeIQx formation. In addition, they also indicated that the 1:1 ratio between acrolein and creatinine was the most appropriate ratio for MeIQx formation.

Although MeIQx was produced when acrolein and creatinine were heated, addition of other compounds produced changes in the reaction yields. Assayed compounds were methylglyoxal, ammonia, and formaldehyde.

Figure 75 shows the addition of methylglyoxal. Addition of small amounts of methylglyoxal (10 μmol to a mixture of 50 μmol of acrolein and 50 μmol of creatinine) increased the amount of produced MeIQx by 30%. On the other hand, higher amounts of methylglyoxal decreased the amount of MeIQx produced, which suggested that an excess of methylglyoxal might compete with acrolein for the amino groups and inhibit MeIQx formation in that way.

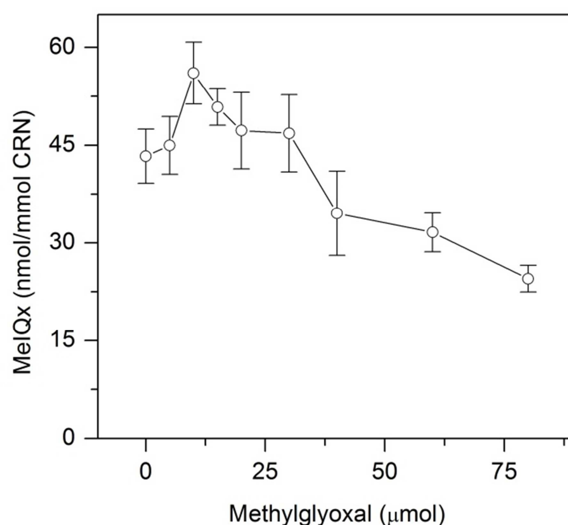


Figure 75. Effect of methylglyoxal addition on MeIQx formation. Abbreviation: CRN, creatinine.

Figure 76 summarizes the effect of the addition of ammonia and formaldehyde on MeIQx formation. As observed in **Figure 76A** and **Figure 76B**, addition of ammonia and formaldehyde to a mixture of 50 μmol of acrolein and 50 μmol of creatinine decreased the amount of MeIQx produced. Thus, addition of ammonia decreased linearly the produced MeIQx both in the absence (r^2 = 0.92, p = 0.008) and in the presence (r^2 = 0.90, p = 0.01) of methylglyoxal (**Figure 76A**). Something similar occurred with the addition of formaldehyde (**Figure 76B**). Thus, the amount of produced MeIQx decreased linearly when the amount of formaldehyde increased both in the absence (r^2 = 0.92, p = 0.008) and in the presence (r^2 = 0.996, $p < 0.0001$) of methylglyoxal. Nevertheless, addition

of small amounts of formaldehyde to the equimolecular mixture of acrolein and creatinine did not exhibit this decrease (**Figure 76B**). Similar results were also observed when ammonium chloride and formaldehyde were added simultaneously (data not shown). However, some of these effects were different when these compounds were added to other mixtures of acrolein and creatinine.

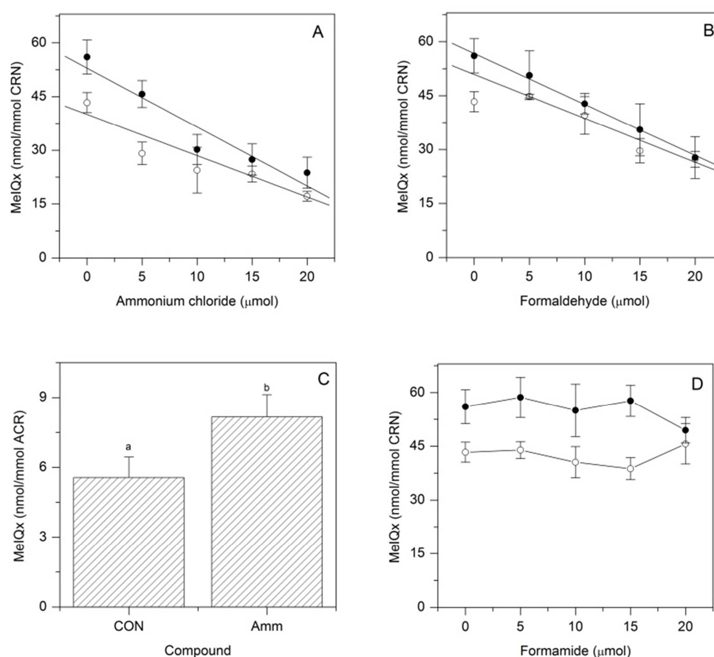


Figure 76. Effect of addition of: A, ammonium chloride; B, formaldehyde; C, ammonium chloride; and D, formamide, in mixtures of acrolein/creatinine (○) and acrolein/creatinine/methylglyoxal (●). Abbreviations: ACR, acrolein; Amm, ammonium chloride; CON, control; CRN, creatinine.

As an example, **Figure 76C** shows the effect of the addition of 10 µmol of ammonium chloride to a mixture containing 50 µmol of acrolein and 10 µmol of creatinine. As observed, the addition of ammonium chloride increased the amount of MeIQx produced.

In addition to formaldehyde and ammonia, the effect of formamide was also assayed because it has been suggested to play a role in the ring closure of PhIP

(Zhang *et al.*, 2020). However, and contrarily to ammonia and formaldehyde, formamide does not seem to take part in the reaction in anyway (**Figure 76D**). Thus, the amount of produced MeIQx was always the same when formamide was added in the range 0–20 μmol. Results shown in **Figure 76D**, suggest that formamide does not play any role in MeIQx formation: Neither formamide increases MeIQx formation nor it seems to react with any of the reactants or intermediates that play a role in MeIQx formation.

Water content also played a major role on the amount of MeIQx produced (**Figure 77**).

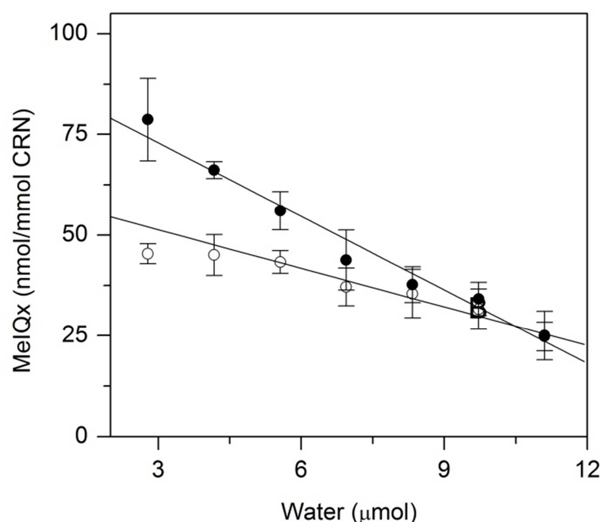


Figure 77. Effect of addition of water on MeIQx formation in mixtures of acrolein/creatinine (○) and acrolein/creatinine/methylglyoxal (●). Abbreviation: CRN, creatinine.

In the absence of methylglyoxal, addition of 3–6 μmol of water always produced approximately the same amount of MeIQx. However, this amount decreased linearly ($r^2 = 0.982$, $p = 0.00097$) when the amount of water increased in the range 6–11 μmol, therefore suggesting that, analogously that described in sections 4.2.1.2 and 4.2.2.2 for MeIQ and IQ, respectively, a high water activity did not favour the formation of MeIQx. This effect was observed more clearly in

the presence of methylglyoxal (**Figure 77**). In this case, the amount of produced MeIQx decreased linearly ($r^2 = 0.984$, $p < 0.0001$) when the amount of added water increased in the range 3–11 μmol.

MeIQx formation also depended on heating time and temperature. It was observed both in presence and in the absence of methylglyoxal (**Figure 78** and **Figure 80**, respectively). As observed in **Figure 78**, the amount of produced MeIQx increased linearly ($r^2 > 0.94$, $p < 0.0001$) when an equimolecular mixture of acrolein and creatinine was heated at temperatures in the range 140–200 ºC.

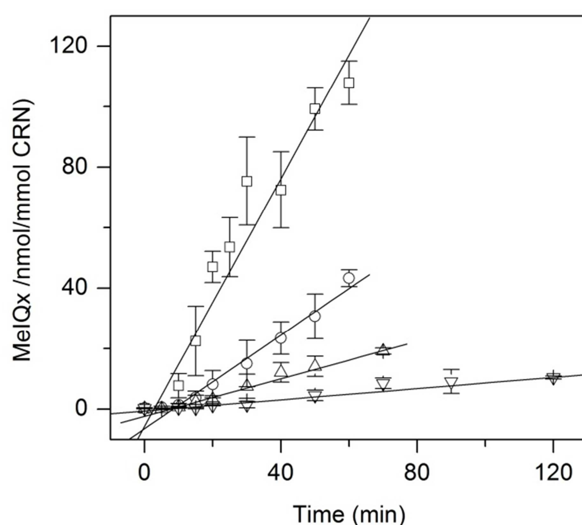


Figure 78. Effect of time and temperature on the formation of MeIQx in mixtures of acrolein/creatinine. Assayed temperatures were: 200 (□), 180 (○), 160 (△), and 140 (▽) ºC. Abbreviation: CRN, creatinine.

In addition, reaction rates, which were determined from the slopes of the obtained lines, increased with temperature. When the natural logarithms of these reaction rates were plotted against the inverse of the absolute temperatures in an Arrhenius plot (**Figure 79**), a linear correlation was observed. The activation energy of MeIQx formation was obtained from the slope of the curve and resulted to be 81.1 ± 1.4 kJ/mol.

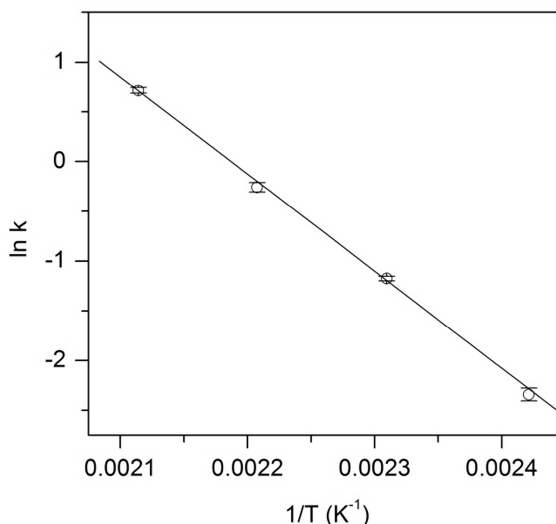


Figure 79. Arrhenius plot obtained for MeIQx formation in mixtures of acrolein/creatinine.

The activation energy of MeIQx is closed to those of 80.9 kJ/mol determined for PhIP (Zamora *et al.*, 2013), 72.2 kJ/mol determined for MeIQ in subsection 4.2.1.2, and 77.0 kJ/mol determined for IQ in subsection 4.2.2.2, which suggests that activation energies for the formation of all these heterocyclic aromatic amines are quite similar.

Formation kinetics of MeIQx in mixtures of acrolein, creatinine, and methylglyoxal were also studied (**Figure 80**). As observed in **Figure 80**, the amount of MeIQx increased linearly ($r^2 > 0.90$, $p < 0.0001$) as a function of time at the four studied temperatures. Reaction rates at the different assayed temperatures were calculated and these rates were employed in an Arrhenius plot to determine the activation energy of MeIQx formation in the presence of acrolein, creatinine, and methylglyoxal (**Figure 81**). The activation energy obtained was 80.6 ± 1.7 kJ/mol, which is not significantly ($p < 0.05$) different to the value of 81.1 ± 1.4 kJ/mol obtained for MeIQx formation in mixtures of

acrolein and creatinine. These results suggest that methylglyoxal does not take part in the limiting step of MeIQx formation.

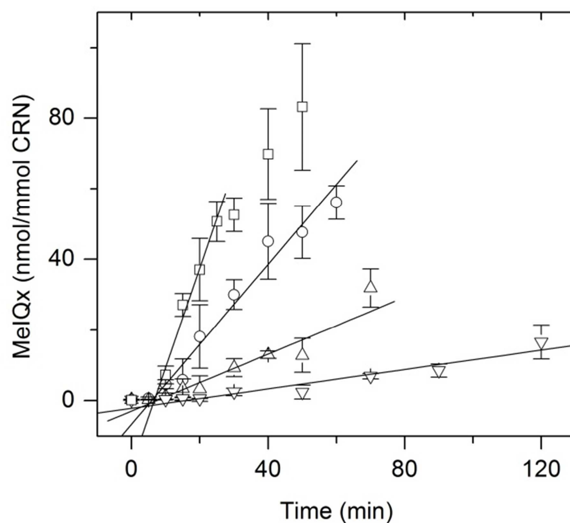


Figure 80. Effect of time and temperature on formation of MeIQx in mixtures of acrolein/creatinine/methylglyoxal. Assayed temperatures were: 200 (□), 180 (○), 160 (△), and 140 (▽) ºC. Abbreviation: CRN, creatinine.

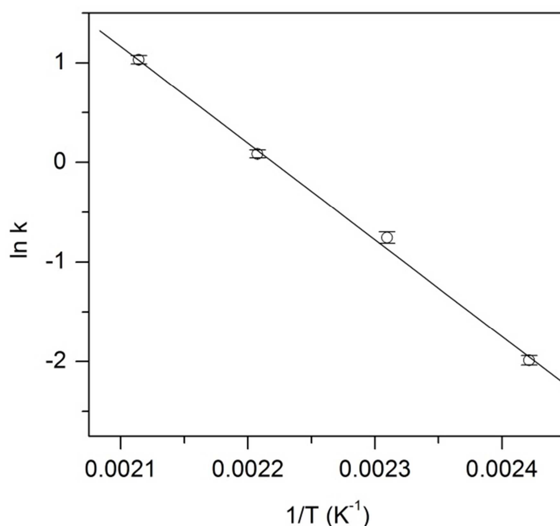


Figure 81. Arrhenius plot obtained for MeIQx formation in mixtures of acrolein/creatinine/methylglyoxal.

4.2.3.2. Formation of MeIQx in reaction mixtures of acrolein-producing compounds and creatinine

MeIQx is not only produced in the presence of acrolein, but also when creatinine was heated in presence of acrolein-producing compounds. As acrolein-producing compounds, oxidized menhaden and linseed oils, 13-hydroxide of linoleic acid, glucose, fructose, ribose, methionine, and threonine were assayed (**Figure 82**).

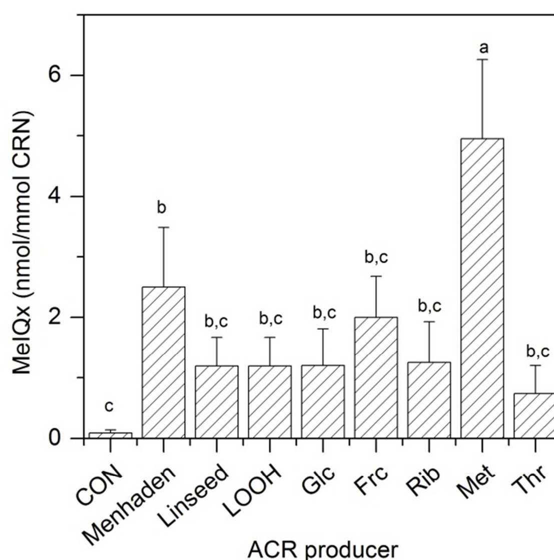


Figure 82. Effect of different acrolein producers on MeIQx formation in mixtures of acrolein producer and creatinine. Assayed acrolein producers were: Menhaden and linseed oils, 13-hydroperoxide of linoleic acid (LOOH), glucose (Glc), fructose (Frc), ribose (Rib), methionine (Met), and threonine (Thr). Mean values with a different letter are significantly ($p < 0.05$) different. Other abbreviations: ACR, acrolein; CRN, creatinine; CON, control, it corresponds to creatinine heated in the absence of acrolein producer.

Although all assayed compounds seemed to produce more MeIQx than control, the observed increase was only significant ($p < 0.05$) for two of them: Methionine and oxidized menhaden oil. These results suggest that acrolein-producing compounds are also able to produce MeIQx.

5. DISCUSSION

Reactive carbonyls are known to contribute to the flavour of foods, such as the formation of Strecker aldehydes discussed in section 1.6.3.1. However, obtained results show that, in the presence of ammonia and ammonia-producing compounds, reactive carbonyls also have a tendency to evolve into heterocyclic derivatives. Produced compounds not only play a role in food aroma but also in food safety.

5.1. Formation of pyridines by cyclization/oligomerization of reactive carbonyls in the presence of ammonia and ammonia-producing compounds

Maillard reaction is recognized as the most important route for the formation of reactive carbonyls, which contribute to flavour generation by carbonyl-amine reactions (Liu *et al.*, 2019; Zhao *et al.*, 2019). However, other major food components also produced reactive carbonyls, such as lipids (Hidalgo & Zamora, 2016) or phenolics (Delgado *et al.*, 2015). Therefore, they are also able to contribute to flavour generation by carbonyl-amine reactions. Due to this variety of origins, many times it is difficult to know what reactive carbonyl is involved in the formation of a specific flavour. This is specially complex in the case of pyridines. The role of both carbohydrates (Paravisini & Peterson, 2019) and lipids (Farmer & Mottram, 1990; Horiuchi *et al.*, 1998) has been suggested. However, the results shown in section 4.1.1 suggest that alkylpyridines are mainly produced as a consequence of cyclization/oligomerization reactions of reactive carbonyls in the presence of ammonia and ammonia-producing compounds.

5.1.1. Effect of ammonia and ammonia-producing compounds on pyridine formation

As described in section 4.1.1.1, the heating of lipid-derived reactive carbonyls in the presence of ammonia and ammonia-producing compounds generated a

wide range of alkylpyridines, including 2-alkylpyridines, 3-alkylpyridines, 2,5-alkylpyridines, and 2,6-alkylpyridines.

The produced pyridines were always the same for the different ammonia-producing compounds assayed, although reaction yields depended on the involved ammonia-producing compound, which suggests that ammonia is the compound responsible for these cyclization/oligomerization reactive carbonyls. In fact, ammonia is produced by thermal decomposition of all assayed compounds upon heating (Chen *et al.*, 2000; Riha *et al.*, 1996; Zamora *et al.*, 2014). Thus, the highest amount of pyridines was produced in the presence of ammonia and urea, followed to glutamine and ammonium chloride. Creatinine was the assayed compound that produced the lowest amount of pyridines. This behaviour is likely a consequence of both, the different ability of producing ammonia among the assayed compounds and the ability of some assayed compounds to produce other reactions with the carbonyl compounds. Thus, the formation of carbonyl-amine adducts been described between reactive carbonyls and amino acids (Zamora & Hidalgo, 2005).

5.1.2. Proposal of a reaction pathway for the formation of 2-alkylpyridines

As shown in section 4.1.1.2, 2-alkylpyridines are mainly produced in mixtures of alkanals and 2-alkenals in the presence of ammonia. In particular, 2-methylpyridine had as precursors acetaldehyde, crotonaldehyde, and 2,4-hexadienal, most likely because these carbonyls are interconverted among them.

Figure 83 shows a general scheme containing two alternative routes for 2-methylpyridine formation. The highest yield for 2-methylpyridine formation was observed in mixtures of acetaldehyde and crotonaldehyde. Acetaldehyde, which can also be produced as a consequence of alanine degradation, is converted into crotonaldehyde be means of an aldol condensation (and, in the same way, the aldol condensation of crotonaldehyde with acetaldehyde produces 2,4-hexadienal). When ammonia was present in the reaction, it can be added to

crotonaldehyde in a first step. Then, the formed amine can produce the corresponding imine with acetaldehyde. A cyclization of this imine, followed by dehydration and oxidation produced 2-methylpyridine.

A proof for the proposed reaction pathway was obtained when deuterated water was employed in the place of water. This change produced the formation of a dideuterated 2-methylpyridine. As shown in **Figure 83**, formation of this pyridine is explained by deuteration of interchangeable protons during ring formation.

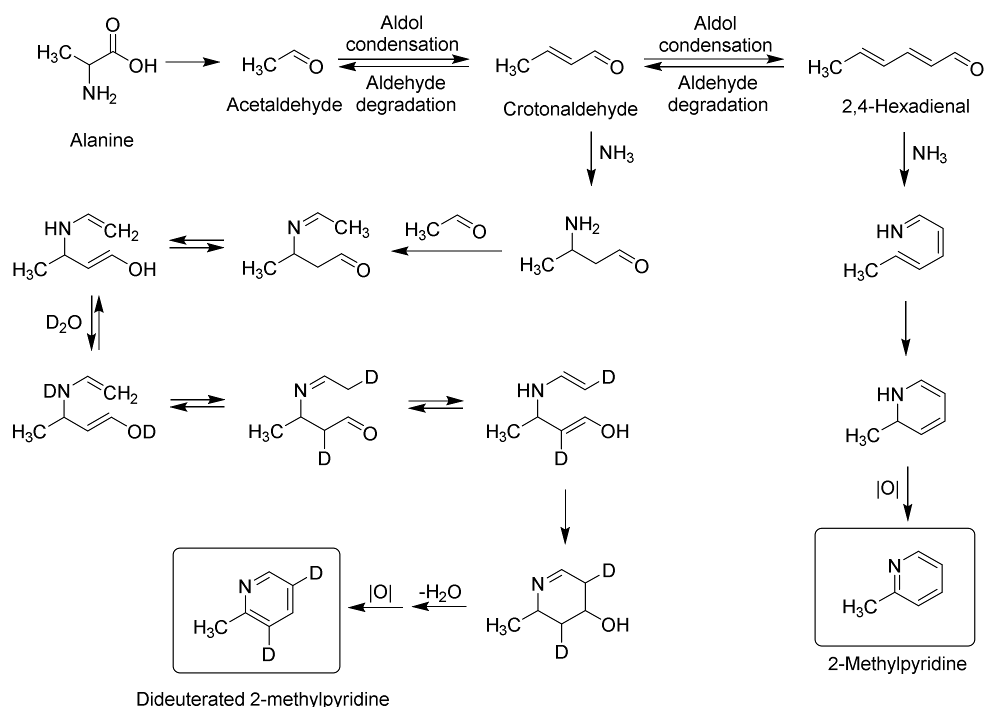


Figure 83. Proposed pathways for the formation of 2-methylpyridine from 2,4-hexadienal or crotonaldehyde/acetaldehyde in the presence of ammonia (or ammonia-producing compounds). Figure also shows the deuterated pyridine produced in presence of deuterated water.

2-Alkylpyridines can also be produced by cyclization of 2,4-alkadienals. Thus, 2,4-hexadienal can produce the corresponding imine with ammonia. Cyclization of this adduct is responsible for the formation of 2-methylpyridine.

Similar reaction mechanisms can be suggested for the formation of other 2-alkylpyridines from the corresponding 2,4-alkadienals or mixtures of 2-alkenals and acetaldehyde in the presence of glutamine or other ammonia-producing compounds. As observed in section 4.1.1.2, there was a decrease in the amount of pyridine produced when the chain length of the reactive carbonyl increased. This is likely a consequence of the different reactivity of the assayed reactive carbonyls because of the carbon chain length.

5.1.3. Proposal of a reaction pathway for the formation of 3-alkylpyridines

3-Alkylpyridines were mainly produced in presence of acrolein and alkanals. **Figure 84** shows a reaction pathway that explains the formation of 3-methylpyridine when acrolein and propanal were heated in the presence of glutamine. The reaction would be initiated by the addition of ammonia to acrolein and the later formation of an imine with propanal. The cyclization of the produced adduct would be the origin of the 3-methylpyridine after dehydration and oxidation.

Analogously to the above described confirmation of the reaction pathway responsible for the formation of 2-alkylpyridines, a proof for this pathway was obtained when water was replaced by deuterated water. In this case, the mono-deuterated 3-alkylpyridine was mainly produced. Formation of this pyridine is again explained by deuteration of interchangeable protons during ring formation.

2,4-Hexadienal also contributed to the formation of 3-methylpyridine. This ability might be related to the described decomposition of the 2,4-alkadienal into acetaldehyde (Zamora *et al.*, 2015b) and into acrolein (Ewert *et al.*, 2014). In fact, mixtures of acrolein/acetaldehyde or acrolein/alanine (alanine is a precursor of acetaldehyde) produced 3-methylpyridine to a significant extent. However, a reaction pathway for the formation of 3-methylpyridine in mixtures of acrolein/acetaldehyde cannot proposed at this time without additional experiments.

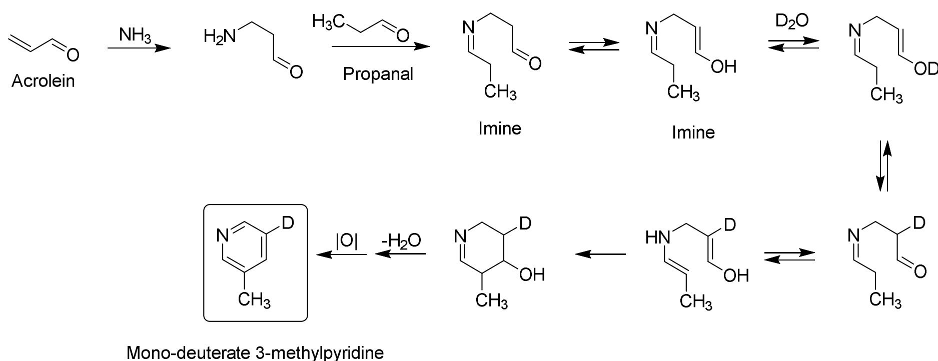


Figure 84. Proposed pathway for the formation of 3-methylpyridine from acrolein and propanal. Figure also shows the deuterated pyridine produced in the presence of deuterated water.

5.1.4. Proposal of a reaction pathway for the formation of 2,5-dialkylpyridines

2,5-Dialkylpyridines were mainly produced when mixtures of 2-alkenals and alkanals were heated in the presence of ammonia-producing compounds. Thus, when propanal and crotonaldehyde were heated in the presence of glutamine, 2,5-dimethylpyridine was produced (**Figure 85**). Analogously to the pathway proposed above, the reaction is suggested to be initiated by addition of ammonia to crotonaldehyde to produce 3-aminopropanal. This amino-carbonyl compound would then react with propanal to produce the corresponding imine. The later cyclization of this adduct would be the origin of 2,5-dimethylpyridine, after dehydration and oxidation, analogously to the above described for other pyridines. Similarly to those studies, this pathway was confirmed by deuterium-labelling experiments. Thus, when water was substituted by deuterated water, dideuterated 2,5-dimethylpyridine was formed. As indicated in **Figure 85**, the formation of this pyridine is explained by deuteration of interchangeable protons during ring formation.

2,5-Dimethylpyridine were also produced in presence of acetaldehyde and in mixtures of crotonaldehyde and 2,4-heptadienal. As indicated in **Figure 85**,

acetaldehyde is a precursor of crotonaldehyde and 2,4-heptadienal is a precursor of propanal.

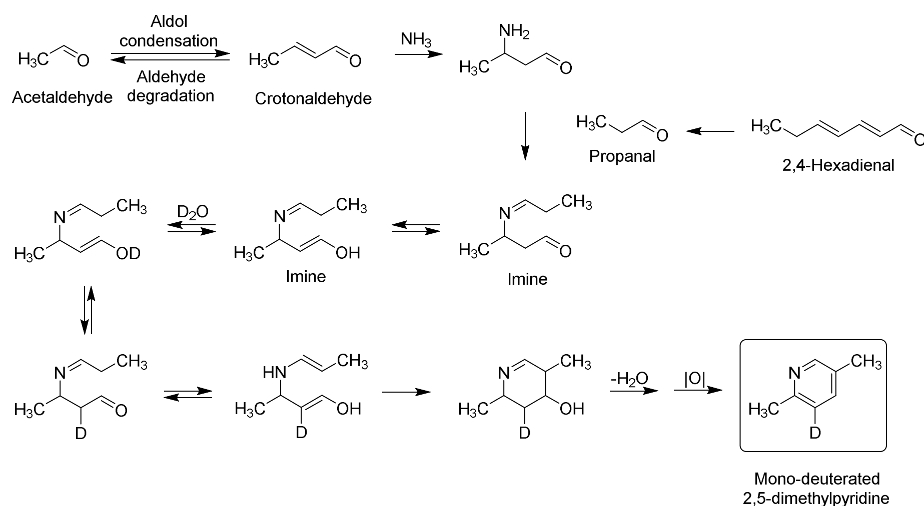


Figure 85. Proposed pathway for the formation of 2,5-dimethylpyridine from crotonaldehyde and propanal. Figure also shows the deuterated pyridine produced in presence of deuterated water.

As expected, according to the proposed reaction pathway, when propanal was substituted by 2-pentenal, 2,5-diethylpyridine was formed. In this case, ammonia is proposed to be firstly added to 2-pentenal and the amine produced reacts then with crotonaldehyde producing the corresponding imine in a first step and, after cyclization, 2,5-diethylpyridine.

The proposed general pathway in **Figure 85** is also valid for the formation of 2,5-dialkylpyridines with different substituents. These heterocyclic derivatives were produced to a high extent by oligomerization and cyclization of 2-alkenals in the presence of ammonia. As an example, **Figure 86** shows the proposed pathway for the formation of 2-ethyl-5-methylpyridine by oligomerization/cyclization of crotonaldehyde. As observed in the formation of other pyridines, the reaction is proposed to be initiated by addition of ammonia to crotonaldehyde, followed by the formation of the imine with a second molecule

of aldehyde. An electronic rearrangement and a dehydration step would be responsible for 2-ethyl-5-methylpyridine formation. Differently to above described pathways, this pathway only needs the presence of one reactive carbonyl and there is not an oxidation step. These may be the main reason for the production of these pyridines to a much higher extent than other described pyridines. This pathway is also valid for the formation of 2,5-dialkylpyridines from other 2-alkenals, including the formation of 2-ethyl-5-propylpyridine from 2-pentenal, 5-butyl-2-propylpyridine from 2-hexenal, 2-butyl-5-pentylpyridine from 2-heptenal, and 5-hexyl-2-pentylpyridine from 2-octenal.

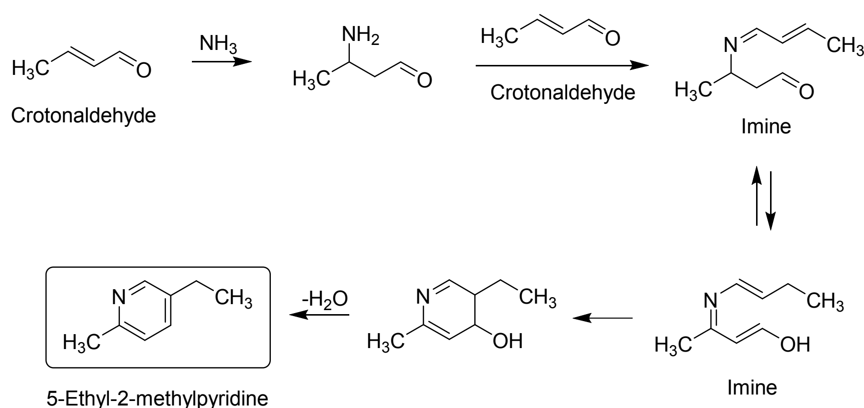


Figure 86. Proposed pathway for the formation of 5-ethyl-2-methylpyridine by oligomerization of crotonaldehyde in the presence of glutamine.

5.1.5. Proposal of a reaction pathway for the formation of 2,6-dialkylpyridines

Differently to other pyridines, 2,6-dialkylpyridines were not a product of the cyclization of short chain aldehydes produced in the lipid oxidation pathway. In fact, they were produced by cyclization of unsaturated ketones also produced in the lipid oxidation pathway. Thus, 2,6-dimethylpyridine was mainly produced by cyclization of 3,5-heptadien-2-one in the presence of glutamine. The proposed formation pathway is shown in **Figure 87**. The reaction can be initiated either by formation of the corresponding imine with ammonia and then cyclization, or by addition of ammonia to the γ,δ carbon–carbon double bond and then, formation

of the imine. In any case, a later oxidation of the produced cyclic intermediate would be responsible for the formation of 2,6-dimethylpyridine.

Analogously to previous reactions, deuterium-labelling experiments were also carried out. In this case, several isomers with different degree of deuteration were produced. As indicated in the **Figure 87**, this time the hydrogen atoms of methyl groups were also interchangeables and deuteration of aromatic protons at positions 3 and 5 can also occurs. This explains the appearance of a significant number of derivatives, some of them with a high deuteration degree. Thus, when the reaction was carried out in the presence of deuterated water, mono- and di-deuterated 2,6-dimethylpyridines were detected to a high extent. In addition, small amounts of tri-deuterated 2,6-dimethylpyridine were also produced.

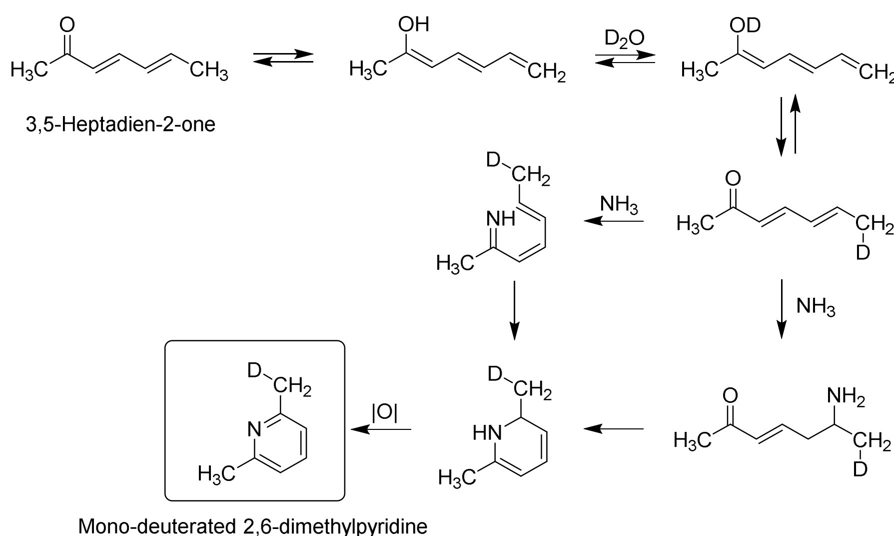


Figure 87. Proposed pathway for the formation of 2,6-dimethylpyridine from 3,5-heptadien-2-one. When the reaction is carried out in the presence of D_2O, several pyridines with different degree of deuteration were produced.

This reaction pathway is also valid for the formation of 2,6-dimethylpyridine from 6-methyl-5-hepten-2-one. However, in this case the hydrogen atom at position 6 in the ketone chain is absent. Therefore, to close ring, the methyl group at position 6 of the ketone chain must leave.

5.1.6. Formation of pyridines in oxidized menhaden oil heated in presence of glutamine

To confirm that alkylpyridines were produced as a consequence of the lipid oxidation pathway, oxidized menhaden oil was heated in the presence of glutamine. As expected, diverse pyridines were produced. The main pyridines detected were 3-methylpyridine and, to a lower extent, 2-ethylpyridine. This is likely related to the easiness of the formation of precursors as a consequence of lipid oxidation. Thus, acrolein, which is a major lipid oxidation product in the oxidation of edible oils, including menhaden oil, and 2,4-heptadienal are easily produced as a consequence of ω3 fatty acyl chain breakage (Nogueira *et al.*, 2019; Shibata *et al.*, 2018). Therefore, they mainly reacted with the ammonia produced as a consequence of the thermal decomposition of glutamine and formed the corresponding pyridines. These results confirm the relationship between lipid oxidation and pyridine formation in food products submitted to thermal heating.

5.2. Formation of 3-hydroxypyridines in foods

Although to a lower extent than the above described alkylpyridines, 3-hydroxypyridines are also produced in food products as a consequence of processing. Its origin was previously unknown. However, results described in sections, 4.1.2 and 4.1.3 show that these pyridines are produced in the lipid oxidation and the Maillard reaction pathways.

5.2.1. Formation of 3-hydroxypyridines as a consequence of lipid oxidation

5.2.1.1. Role of lipid-derived reactive carbonyls on 2-alkyl-3-hydroxypyridines formation

Results described in section 4.1.2 show that 3-hydroxypyridines can be produced as a consequence of lipid oxidation by cyclization of reactive carbonyls in the presence of ammonia. In addition, these results showed that the reactive

carbonyls responsible for the production of 3-hydroxypyridines were 4,5-epoxy-2-alkenals. Furthermore, 2,4-alkadienals also produced 3-hydroxypyridines, but an oxidation was required (the reaction was not produced under an inert atmosphere). Moreover, the amount of pyridines produced by both of them under air was quite similar. Thus, 2,4-alkadienals should be oxidized and converted into epoxyalkenals as a previous step to its cyclization to produce the corresponding pyridine. Having into account that 2,4-alkadienals are common lipid oxidation products (Beltrán *et al.*, 2011; Zhao *et al.*, 2019), these results suggest that 3-hydroxypyridines should be produced in foods when lipids are oxidized.

3-Hydroxypyridines formation is not the only carbonyl-amine reaction in which 2,4-alkadienals are involved. Thus, for example, they have been described to be involved in Strecker-type degradation (Zamora *et al.*, 2007) or amino acid decarboxylation (Hidalgo *et al.*, 2016; Zamora *et al.*, 2015a). Therefore, all these reactions compete among them and reaction conditions play a major role on the produced compounds. In addition, 2,4-alkadienal chain length also influenced reaction yield. Thus, the highest yield was achieved with 2,4-hexadienal, and it decreased when the chain length of the alkadienal increased.

5.2.1.2. *Role of ammonia and ammonia-producing compounds on 3-hydroxypyridines formation*

Analogously to that observed in alkylpyridines, the type of 3-hydroxypyridines produced depended on the lipid-derived carbonyl compound involved and not on the type of amino compound, because the same pyridine was always produced with the different ammonia-producing compounds assayed. However, the amount of pyridine changed according to the ammonia-producing compound involved. This is likely related to the easiness of producing ammonia by heating of assayed compounds. Thus, the hydroxypyridine was produced in a decreasing order by ammonia, ammonium chloride, glutamine (which is also a good ammonia producer as found by Kim & Ho, 1998b), urea, alanine, and creatinine. Other important factor that also plays a role to this respect is the

ability of the amino compound to produce carbonyl-amine reactions. In fact, amino acids react easily with lipid-derived reactive carbonyls to produce both carbonyl-amino acid adducts (Zamora & Hidalgo, 2005), and amino acid degradations (Hidalgo & Zamora, 2016). Therefore, the best ammonia-producer and the worst carbonyl scavenger would be the ammonia-producing compound that produce pyridines to the higher extent. This occurs with ammonia and ammonia chloride.

5.2.1.3. 3-Hydroxypyridines formation pathway

A pathway for the formation of 2-alkyl-3-hydroxypyridines is proposed in **Figure 88**. As shown in **Figure 88**, the reaction can take place with corresponding 4,5-epoxy-2-alkenals, but can also with 2,4-alkadienals. This last compounds are easily converted into 4,5-epoxy-2-alkenals by means of an oxidative process.

The reaction between 4,5-epoxy-2-alkenals and ammonia can take place with the production of the corresponding imine. Formation of imines in the reaction between epoxyalkenals and amino compounds was observed previously (Hidalgo & Zamora, 1993). The cyclization of the imine would produce a dihydropyridine. This compound can follow two alternative pathways. One of them is its aromatization to produce the corresponding 2-alkyl-3-hydroxypyridine. Alternatively, the dihydropyridine can suffer a dehydration and be converted into 2-alkylpyridine. Alkylpyridines were also detected in the assayed reaction mixtures, but only at trace amounts. 2-Alkyl-3-hydroxypyridines were the main products of the reaction. 2-Alkylpyridines were mainly produced by following the reaction pathway described in section 5.1.2.

As described in section 4.1.2, in addition to the lipid-derived reactive carbonyls and the ammonia-producing compounds, formation of 3-hydroxypyridines also depended on reaction time, temperature, and pH. Proposed pathway in **Figure 88** explains obtained results. Thus, the amount of pyridine

remained more or less unchanged between pH 7 and 8. However, higher basicity inhibited the reaction, most likely because it induced the degradation of the 2,4-alkadienal (Zamora *et al.*, 2015b). In addition, 3-hydroxy-2-methylpyridine seemed to be produced to a higher extent in the presence of phosphate buffer than in the presence of borate buffer, most likely because the presence of phosphate buffer facilitates the oxidation of the 2,4-alkadienal and the formation of the imine required to produce the 2-alkyl-3-hydroxypyridines instead of other carbonyl-amine reactions.

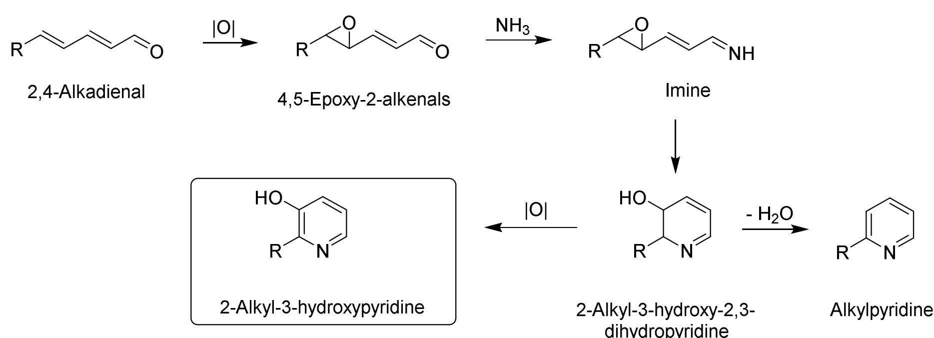


Figure 88. Proposed pathway for the formation of 2-alkyl-3-hydroxypyridines from 2,4-alkadienals and 4,5-epoxy-2-alkenals.

This reaction pathway also explains the formation of 3-hydroxypyridine from 4-pentenal (**Figure 89**). The only difference with the pathway described for 2-alkyl-3-hydroxypyridines formation from 2,4-alkadienals (**Figure 88**) is that a second oxidative step is needed to produce 3-hydroxypyridine. Thus, the oxidation of 4-pentenal would produce the corresponding epoxyalkanal. This last compound would be able to react with ammonia to produce the imine in a first step and, after cyclization, a tetrahydropyridine. This compound would need two oxidation steps to produce 3-hydroxypyridine, which would explain the much lower reaction yield for the formation of 3-hydroxypyridine from 4-pentenal in relation to the reaction yields obtained for the conversion of 2,4-alkadienals into 2-alkyl-3-hydroxypyridines.

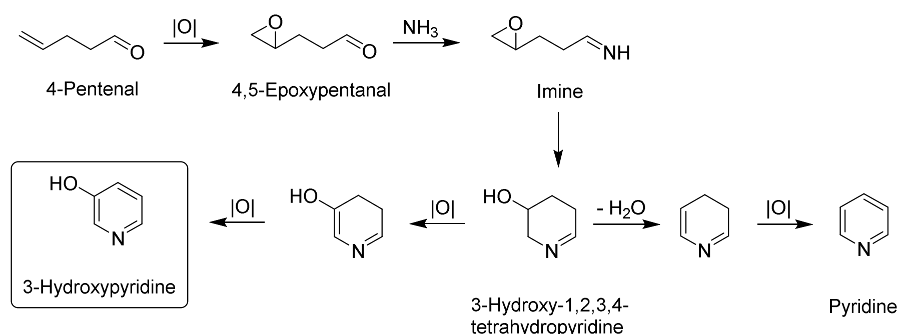


Figure 89. Proposed pathway for the formation of 3-hydroxypyridine from 4-pentenal.

5.2.1.4. Formation of 3-hydroxypyridines in oxidized oils heated in presence of glutamine

To confirm that these pyridines were produced as a consequence of the lipid oxidation pathway, oxidized linseed and menhaden oils were heated in the presence of glutamine. As expected, both oils produced 3-hydroxypyridines. However, because they have different fatty acyl residues, the amount of the produced 3-hydroxypyridines were different among them. This is likely related to the different ability of fatty acyl chains to produce lipid-derived reactive carbonyls, which are produced to different extents and proportions (Liu *et al.*, 2020). Thus, linseed oil has a higher linolenic acid content than menhaden oil (Sebedio & Ackman, 1983), and 2,4-hexadienal is produced to a higher extent (Kiralan *et al.*, 2018). This explains the higher formation of 3-hydroxy-2-methylpyridine in linseed oil in relation to that produced in menhaden oil. Similarly, 2,4-heptadienal, which is an aldehyde derived from ω3 rich oils, is the precursor of 2-ethyl-3-hydroxypyridine, which was also produced to a significant extent. On the other hand, 3-hydroxypyridine was produced to a higher extent by oxidized menhaden oil than by linseed oil.

5.2.2. Formation of 3-hydroxypyridines as a consequence of Maillard reaction in foods

Lipids are not the only source for 3-hydroxypyridines in foods. The results collected in section 4.1.3 show that these pyridines can also be produced as a consequence of Maillard reaction from the 2-oxofurans produced in the Maillard pathway.

5.2.2.1. Proposal of a reaction pathway for 3-hydroxypyridines formation by ring expansion of 2-oxofurans

2-Oxofurans are common products in the Maillard reaction pathway. In particular, 5-hydroxymethylfurfural (HMF) is a marker of Maillard reaction because it is produced to a high extent in many food products.

Diverse chemical syntheses have suggested that conversion of HMF into 2-hydroxymethyl-5-hydroxypyridine is possible (Müller *et al.*, 1998). However, this is first time in which the conversion of 2-oxofurans into 3-hydroxypyridines is shown under conditions close to those employed in food processing. The reaction is proposed to take place as described in **Figure 90**. Thus, in the presence of ammonia, the corresponding imine should be produced in a first step. Then, the addition of a second molecule of ammonia would produce the opening of the ring with the formation of a compound derived from the dicarbonyl precursor of the furan. The attack of the second molecule of ammonia is likely a consequence of the polarization of the C–O bond in the furan ring, which is a consequence of the difference of electronegativities between both atoms. Finally, cyclization of this intermediate with the exit of a molecule of ammonia would produce the corresponding hydroxypyridine.

Because the reaction only requires the presence of an oxo group at position 2 of the furan ring, any other 2-oxofuran that might be present should also suffer the reaction. In addition, it could also expected to be produced in analogous 2-

oxoheterocycles, such as 2-acetylthiophene (Begum *et al.*, 2019; Dong *et al.*, 2019).

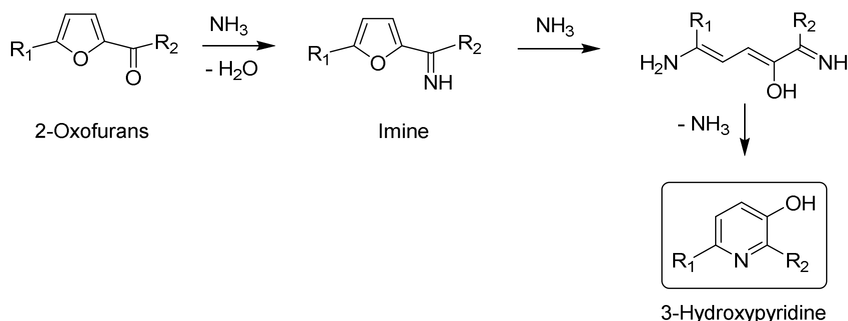


Figure 90. Proposed pathway for the formation of 3-hydroxypyridines from 2-oxofurans. For furfural and 3-hydroxypyridine, R_1=H and R_2=H. For 2-acetylfuran and 3-hydroxy-2-methylpyridine, R_1=H and R_2=CH_3. For HMF and 2-hydroxymethyl-5-hydroxypyridine, R_1=CH_2OH and R_2=H.

Reaction yield depended on the existence of substituents in the carbon at position 5 of the furan ring. The hydroxyl group of the hydroxymethyl substituent at position 5 of HMF has a negative inductive effect. This effect should contribute to increase the electron deficiency of the carbon at position 5 of the furan ring, therefore converting this atom into a better electrophile. For this reason, HMF is converted more easily into 2-hydroxymethyl-5-hydroxypyridine than furfural into 3-hydroxypyridine.

When activation energies were determinated, activation energy of HMF disappearance was lower than that found for 2-hydroxymethyl-5-hydroxypyridine formation. This explain that HMF can be decomposed (as observed by Chambel *et al.*, 1998) but it is only partially converted in 2-hydroxymethyl-5-hydroxypyridine.

5.2.2.2. Formation of 3-hydroxypyridines in honeys as a consequence of heating

To confirm that the formation of 3-hydroxypyridines by ring expansion of 2-oxofurans occurs in foods, two different honeys were selected and submitted to

thermal treatment. Assayed honeys were a high quality honey, with a very low content of HMF, and a sugarcane honey, which contained a significant content of HMF because of the thermal treatment at which this honey is submitted during its production. In both cases the formation of 2-oxofurans and 3-hydroxypyridines was observed.

In the thermal treatment of the high quality honey, HMF and 2-hydroxymethyl-5-hydroxypyridine formation kinetics were different (section 4.1.3.3). Thus, while HMF concentration increased exponentially as a function of the heating time, the formation of 2-hydroxymethyl-5-hydroxypyridine increased linearly as a function of time. This difference of time courses does not necessarily mean that 2-hydroxymethyl-5-hydroxypyridine is not produced by ring expansion of HMF. As observed in the studied model systems (**Figure 45**), increased amounts of HMF did not always produce increasing amounts of 2-hydroxymethyl-5-hydroxypyridine. This conversion requires the presence of ammonia (or other ammonia-producing compound) that can be the limiting factor in this conversion. In addition, analogously to that observed in model systems, HMF disappearance should produce other compounds in addition to 2-hydroxymethyl-5-hydroxypyridine.

When sugarcane honey was studied, the obtaining results were different. In this case, HMF and 2-hydroxymethyl-5-hydroxypyridine were already present at the initial sample, and the concentration of both of them increased linearly as a function of the heating time. In addition, the 2-hydroxymethyl-5-hydroxypyridine /HMF ratio was higher at 60 °C than at 100 °C, therefore suggesting that decomposition of HMF at low temperature produced the pyridine to a higher extent than at a higher temperature. Therefore, higher temperatures should favour the transformation of HMF into other compounds.

Obtained results suggest that, analogously to that observed in honey, sugarcane honey, and model systems, conversion of 2-oxofurans into 3-hydroxypyridines is unavoidable when 2-oxofurans are submitted to thermal

heating and ammonia (or an ammonia-producing compound) is present. This suggests that, although not frequently found in foods, 3-hydroxypyridines should be common components of foods in which Maillard reaction has occurred, even in those foods submitted to soft heating or stored for a limited time period.

Other consequence of the observed changes in honeys upon thermal treatment might be related to the observed changes in the antioxidant activity of these food products. Thus, 3-hydroxypyridines have been shown to be efficient chain-breaking antioxidants (Kumar *et al.*, 2008). Because their content increases upon heating, the antioxidant activity of honeys should also increase upon heating, as it commonly observed. Therefore, the contribution of 3-hydroxypyridines to the antioxidant activity of heated honeys can be hypothesized. In fact, antioxidant activity of 3-hydroxypyridines have been suggested to be higher than that of α-tocopherol (Kumar *et al.*, 2008). In addition, this antioxidant activity increases when a substituent is present at *para* position of the hydroxyl group, such as in 2-hydroxymethyl-5-hydroxypyridine.

5.3. Formation of heterocyclic aromatic amines by cyclization of reactive carbonyls in the presence of creatinine

Previous sections have shown that, in the presence of ammonia, reactive carbonyls suffer cyclization reactions and the formation of pyridines are produced. This reaction also occurs in the presence of creatinine as ammonia-producing compound. However, in the presence of creatinine, the yield of the produced pyridines was always lower than that observed in the presence of others ammonia-producing compounds. One reason for that is the possibility of creatinine to take part in the cyclization reactions of reactive carbonyls. Obtained results show that creatinine participates in these cyclization reactions and the formation of heterocyclic aromatic amines with structure of aminoimidazoazarenes is produced. This section will discuss the reaction pathways by which these heterocyclic aromatic amines are produced.

5.3.1. Role of reactive carbonyls on the formation of heterocyclic aromatic amines

Analogously to above described reactions conducting to the formation of pyridines, reactive carbonyls are the main compounds responsible for the formation of heterocyclic aromatic amines. Furthermore, the kind of heterocyclic aromatic amine produced is a consequence of the reactive carbonyl involved. Thus, MeIQ is only produced when crotonaldehyde is present in the reaction mixture. Moreover, the amount of MeIQ produced always decreased when other reactive carbonyl was also present, what was likely a consequence of the competition existing among the several carbonyl compounds for the creatinine required for the formation of the heterocyclic aromatic amine.

The origin of crotonaldehyde in foods is unclear. Crotonaldehyde has been described to be produced as a consequence of lipid oxidation by oxidation of ω3 fatty acyl chains in oils and fatty foods (Granvogl, 2014; Papastergiadis *et al.*, 2014; Vieira *et al.*, 2017). In addition, MeIQ was described to be produced in mixtures of creatinine, amino acids, and glucose (Pais *et al.*, 1999), although it has not been observed under reaction conditions employed in this study. These results suggest that crotonaldehyde might have different origins. Thus, previous studies have shown that acetaldehyde is generated from carbohydrates (Talhout *et al.*, 2006) and the conversion of acetaldehyde into crotonaldehyde has been observed, for example, in the production of pyridines in section 5.1.2. Therefore, the contribution of other crotonaldehyde-producing compounds, perhaps through the formation of acetaldehyde, should be considered. Thus, for example, the production of acetaldehyde by the thermal decomposition of serine (Yaylayan *et al.*, 2000) can be the explanation for the special contribution of serine to the formation of MeIQ according to **Table 10**.

Crotonaldehyde, in addition to acrolein, also took part in the formation of IQ. The chemical structure of IQ is very similar to that of MeIQ. They only differ in the methyl group at position 4, which are present in MeIQ and is absent in IQ.

This is the reason for IQ needing the participation of acrolein, which has a chemical structure similar to that of crotonaldehyde but with only three carbon atoms in the place of four carbon atoms of crotonaldehyde. This means that mixtures of crotonaldehyde and acrolein should produce mixtures of MeIQ and IQ, as observed experimentally. In fact, results in section 4.2.2.2 show that the IQ/MeIQ ratio is mostly determined by the acrolein/crotonaldehyde ratio. As observed in **Figure 65**, in the absence of acrolein, the reaction only produces MeIQ. In addition, increasing amounts of acrolein produced an increase in IQ and a decrease in MeIQ. This behaviour is due to a competition between acrolein and crotonaldehyde for the creatinine. Nevertheless, this result was reversed at a high acrolein concentration. Thus, after achieving a maximum with a mixture of 20 μmol of acrolein and 10 μmol of crotonaldehyde, the amount of both IQ and MeIQ decreased afterwards. In fact, to produce a similar amount of both IQ and MeIQ, 25 μmol of acrolein and 10 μmol of crotonaldehyde were required. This might be a consequence of either an easier formation of MeIQ in relation to that of IQ or the involvement of acrolein in further reactions as will be discussed below. To limit this competition, the reaction was carried out in two steps. Firstly, acrolein was incubated with creatinine at a low temperature, and then, crotonaldehyde and glutamine were added, and the reaction mixture was heated at high temperature. The obtained results, showed in section 4.2.2.1, suggested that this first incubation played a major role in the amount of heterocyclic aromatic amines obtained, although the IQ/MeIQ ratio ($\cong$ 0.78) was independent of the incubation time. When mixtures of crotonaldehyde and acrolein were heated in the presence of creatinine, MeIQ seemed to be produced more easily than IQ, although the activation energy for the formation of both heterocyclic aromatic amines was quite similar. In addition, IQ formation decreased at high levels of creatinine (**Figure 66**) which suggested that creatinine might react with some of the intermediates and be involved in further reactions.

One of these reactions is the formation of heterocyclic aromatic amine MeIQx. Thus, when a mixture of acrolein and creatinine was heated, the

formation of MeIQx was observed. Because acrolein is formed by thermal degradation of many molecules, different origins can be hypothesized for MeIQx, including carbohydrates, fats, and amino acids. Thus, acrolein is produced by fragmentation of carbohydrates, most likely as a consequence of the dehydration of hydroxypropanal (Yaylayan *et al.*, 1999). It is also produced from lipids by oxidative degradation (Yin & Porter, 2005), and also from amino acids as a consequence of the Strecker degradation of methionine (Ballance, 1961) and threonine (Anderson *et al.*, 1997).

In addition to acrolein, MeIQx formation also involves other reactive carbonyls. Thus, when methylglyoxal was added, to a low extent, to mixtures of acrolein and creatinine, the amount of the produced MeIQx increased. This suggested that methylglyoxal also took part in the reaction. Nevertheless, addition of methylglyoxal was not required for producing MeIQx, most likely because of the described conversion of acrolein into methylglyoxal (Niyati-Shirkhodaee & Shibamoto, 1993). On the other hand, addition of high amounts of methylglyoxal decreased the MeIQx produced. This suggested that methylglyoxal competes with acrolein for the amine groups present, and an excess of methylglyoxal inhibits the formation of MeIQx.

The effect of addition of other reactive carbonyls on MeIQx formation was also studied. Thus, the effect of formaldehyde was described in section 4.2.3.1. The effect of formaldehyde was studied because it played a key role in the ring closure of PhIP (Zamora *et al.*, 2014). When small amounts of formaldehyde were added, MeIQx formation remained unchanged. However, higher amounts of formaldehyde decreased the MeIQx produced, most likely because of the ability of formaldehyde to react with some of the reactants or intermediates and inhibit the reaction in that way. Thus, formaldehyde, as a reactive carbonyl, is expected to compete with acrolein and methylglyoxal for the existing amino groups, and, therefore, inhibit MeIQx formation. On the other hand, formaldehyde is produced by degradation of acrolein (Zamora *et al.*, 2015b) and also by degradation of

creatinine (Zamora *et al.*, 2014). Therefore, although small amounts of formaldehyde might be needed to complete MeIQx structure, they can be generated in situ and any excess of formaldehyde should decrease the amount of MeIQx produced.

5.3.2. Contribution of ammonia-producing compounds to the formation of heterocyclic aromatic amines with the structure of aminoimidazoazarenes

In addition to the reactive carbonyl and creatinine, ammonia is also required to complete the formation of the structure of the heterocyclic aromatic amines. However, ammonia has not to be added as such. It can be produced by thermal degradation of many compounds. Thus, for example, all amino compounds assayed in section 4.2.1.1, produced MeIQ, although the amount of MeIQ produced depended on the ammonia-producing compound added. Thus, all assayed compounds generated ammonia upon heating (Sharma *et al.*, 2004). However, some ammonia-producing compounds also produced other reactions. Thus, glutamine easily releases free ammonia upon heating (Kim & Ho, 1998a) and the results obtained with this amino acid were similar to the obtained results with ammonia and ammonium chloride in the formation of MeIQ, when small amounts of all of them were added. However, at high concentrations of glutamine, the amount of MeIQ was reduced. This different behaviour at low and high concentrations of glutamine is likely a consequence of different competing reactions that are taken place. Thus, while, at low concentrations, glutamine should contribute by producing the needed ammonia, at high concentrations, glutamine can react with crotonaldehyde to produce carbonyl-amine reactions (Hidalgo & Zamora, 2016; Zamora & Hidalgo, 2005), which would decrease the concentration of the crotonaldehyde required for the formation of MeIQ. This is also likely one of the reasons for the decrease of MeIQ produced when large amounts of crotonaldehyde are present (**Figure 56**): Crotonaldehyde can react with glutamine and decrease the amount of ammonia produced.

Therefore, the formation of heterocyclic aromatic amines is the consequence of the presence of different reactants, but these compounds are also involved in other reactions. Furthermore, some of these compounds can be the origin of others (such as creatinine as a source of ammonia). Therefore, small changes in the concentration of one of these reactants can promote alternative reaction pathways with the formation of other compounds. Thus, for example, the maximum amount of MeIQ was produced with 80 μmol of crotonaldehyde, 20 μmol of creatinine, and 12 μmol of glutamine. Having into account that formation of MeIQ requires (see below) two molecules of crotonaldehyde, one molecule of creatinine, and one molecule of ammonia, the excess of crotonaldehyde required is a consequence of the involvement of this aldehyde in side reactions. In addition to that, the lower ammonia required is a consequence of both being creatinine a source of ammonia, and being ammonia an inducer of the cyclizations of crotonaldehyde to produce pyridines, among other carbonyl-amine products.

Another example of this competition among different reactions was observed when the role of ammonia on MeIQx formation was studied. Thus, while addition of even small amounts of ammonium chloride in the presence of an equimolecular mixture of acrolein and creatinine decreased the MeIQx produced, when ammonium chloride was added to a mixture of 50 μmol of acrolein and 10 μmol of creatinine, the amount of MeIQx increased. In the presence of a high concentration of creatinine, ammonia is produced to a significant extent as a consequence of creatinine thermal decomposition. Therefore, this excess of ammonia is likely to induce the oligomerization/cyclization of acrolein and the production of 3-methylpyridine (as shown in section 5.1.1). On the other hand, when creatinine is the limiting factor, creatinine is needed to produce the heterocyclic aromatic amine and a small amount of ammonia increased the amount of MeIQx

5.3.3. Proposal of reaction pathways for the formation of heterocyclic aromatic amines with the structure of aminoimidazoazarenes

Results described in section 4.2 show that heterocyclic aromatic amines formation requires the presence of reactive carbonyls (crotonaldehyde for MeIQ, crotonaldehyde/acrolein for IQ, and acrolein for MeIQx), together with creatinine, and ammonia. This suggests that, analogously to pyridine formation, these reactions are a consequence of tendency of reactive carbonyls to evolve into cyclic derivatives. Having into account the reactants required for the formation of each heterocyclic aromatic amine identified in section 4.2 and the reaction conditions needed for the formation of these heterocyclic aromatic amines, a reaction pathway for the formation of each of the studied heterocyclic aromatic amines can be proposed.

MeIQ formation only requires the presence of crotonaldehyde, creatinine, and ammonia. The reaction occurs within a wide pH range, but mainly at pH 6–7. High water activity inhibited the reaction, as observed in food products (Jägerstad *et al.*, 1998), and the presence of oxygen is required, although an atmosphere of pure oxygen reduced reaction yield. Finally, activation energy is high and the reaction requires high temperature, as observed in food products (Barzegar *et al.*, 2019). A reaction pathway that is in agreement with all these requirements are proposed in **Figure 91**.

The reaction is suggested to be initiated by addition of creatinine (**2**) to the carbon–carbon double bond of crotonaldehyde (**1**). The produced adduct (**3**) would then react with ammonia to produce the corresponding imine (**4**). A tautomerism would convert this imine into the corresponding enamine (**5**), which can react with a second molecule of crotonaldehyde (**1**) to produce the corresponding imine (**6**). This adduct already contains all atoms present in MeIQ. Only some electronic rearrangements, cyclizations, oxidations, and a dehydration are needed so that the structure of MeIQ can be produced. The leitmotiv of these

reactions is to get an extended conjugation so that the molecule with the most stable structure is produced.

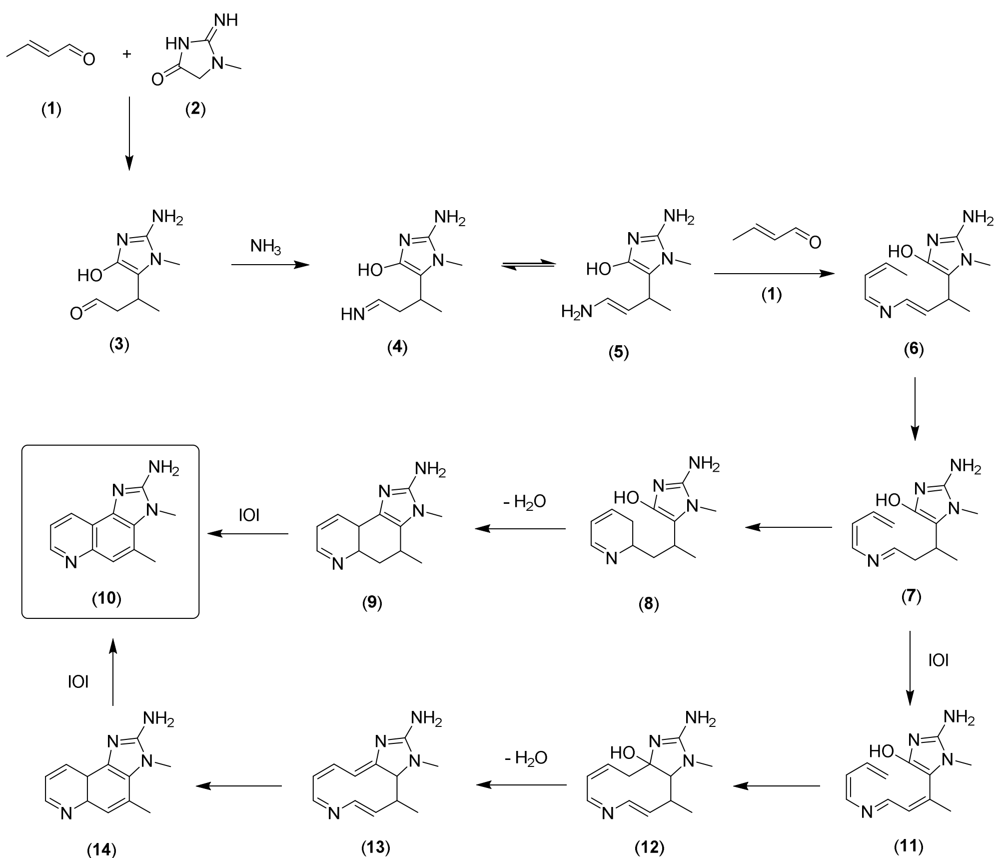


Figure 91. Proposed formation pathway for MeIQ.

Two possible routes are suggested for MeIQ (**10**) formation. The first one starts with the electronic rearrangement of **6** into **7**, followed by a cyclization to produce compound **8**. A dehydration of **8** would produce **9**, which, after oxidation, would produce MeIQ (**10**). The second proposed route is slightly different. It starts by an oxidation of **7** to produce **11**, followed successively by an electronic rearrangement (cyclization) to form **12**, a dehydration to produce compound **13**, and an electronic rearrangement to generate **14**, and an oxidation to finally produce MeIQ (**10**).

This reaction pathway explains that the compound needed to a highest extent is crotonaldehyde (in fact, two molecules of crotonaldehyde per molecule of creatinine and ammonia are needed). However, the maximum amount of MeIQ was not achieved with a 2:1:1 (crotonaldehyde:creatinine:ammonia) ratio. In fact, the maximum amount of MeIQ was produced with 80 µmol of crotonaldehyde, 20 µmol of creatinine, and 12 µmol of glutamine. The reason for this is double: The reactivity of some of the involved reactants and the different routes for formation of other compounds. Thus, crotonaldehyde can suffer numerous reactions including either its oxidation to produce 2-butenoic acid or its degradation to produce acetaldehyde (Zamora *et al.*, 2015b). In addition, ammonia is produced not only by glutamine decomposition but also by thermal decomposition of creatinine. The fact that creatinine is a usual constituent of proteinaceous foods, and ammonia-generating compounds are spreaded out everywhere, suggests that crotonaldehyde is the key reagent for MeIQ formation.

When mixtures of crotonaldehyde, creatinine, and glutamine also contained acrolein, the formation of mixtures of IQ and MeIQ was observed. IQ was produced within a wide pH range, and its formation yield depended on the concentration of precursors, time, and temperature. In addition, to increase the reaction yield, acrolein was incubated with creatinine at low temperature previously to the addition of crotonaldehyde and glutamine and the heating at high temperature. A reaction pathway that explains this behaviour as well as the simultaneous formation of IQ and MeIQ is proposed in **Figure 92**. The reaction is suggested to be initiated by addition of the carbon at position 5 of the creatinine (**2**) ring to the C-3 of acrolein (**15**) to produce the corresponding adduct (**16**). This reaction is produced at a low temperature. However, produced adduct (**16**) is not stable, and reaction can be reversed. Once produced, adduct (**16**) can react with ammonia to produce the corresponding imine (**17**), which is in equilibrium with the enamine form (**18**). This enamine (**18**) can react with crotonaldehyde (**1**) to produce a new imine (**19**). This adduct already has all atoms of IQ (**23**) molecule. However, it has to be stabilized. The suggested route

for the conversion of adduct (**19**) to IQ (**23**) implies electronic rearrangements to produce (**20**) and (**21**), followed by dehydration to form (**22**), and aromatization by which IQ (**23**) is finally produced. As suggested in **Figure 91**, alternative conversions from **19** to **23** are also possible. Thus, an oxidation can be produced followed by dehydration and electronic rearrangements (compounds **6**, **7**, **11**, **12**, **13**, and **10** in **Figure 91**).

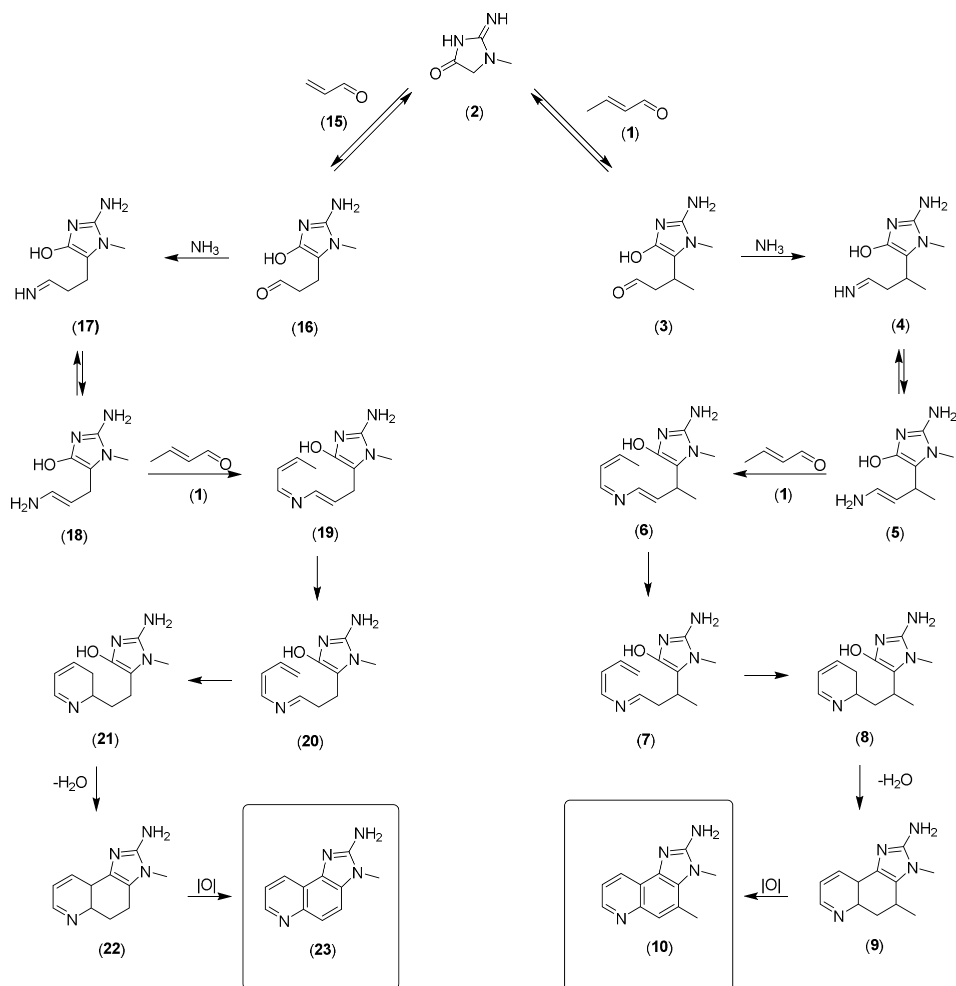


Figure 92. Proposed pathway for the competitive formation of IQ and MeIQ.

The fact that the addition of creatinine (**2**) to acrolein (**15**) is reversible means that, although acrolein (**15**) was added previously to crotonaldehyde (**1**), when

this last aldehyde was added, an alternative route conducting to the formation of MeIQ (**10**) can be produced. In fact, this alternative route should compete with that of IQ (**23**) formation. As indicated in **Figure 92**, both routes are very similar. Thus, once creatinine (**2**) has been added to crotonaldehyde (**1**) to produce adduct (**3**), this adduct would react with ammonia to produce the corresponding imine (**4**) and then the amine (**5**) as a consequence of an imine−enamine tautomerism. The amine (**5**) can react then with a second molecule of crotonaldehyde (**1**) to produce the imine (**6**). Conversion of adduct (**6**) into MeIQ (**10**) is suggested to occur through electronic rearrangements to produce (**7**) and (**8**), followed by dehydration to form (**9**), and, finally, aromatization to produce MeIQ (**6**).

This similarity among the proposed reaction pathways for the formation of both IQ and MeIQ is in agreement with the similarity found for the activation energy of both of them (77.0 ± 1.3 and 72.2 ± 0.4 kJ/mol, for IQ and MeIQ respectively) (**Figure 70** and **Figure 71**) as well as the fact that they had a very similar behaviour in relation to pH (**Figure 64**).

On the other hand, IQ and MeIQ formation did not exhibit identical behaviour in relation to the amount of added water, although their behaviour was quite similar with a maximum at approximately the same water activity (**Figure 67**). Thus, they followed a Gaussian behaviour in relation to the amount of water added. This suggests that formation of these heterocyclic aromatic amines is favoured neither at a high water activity nor under extreme dehydration. Although foods are very complex systems from a chemical and structural point of view, and striking differences are frequently observed between real foods and model systems (Capuano *et al.*, 2018), similar behaviour was found in relation to water activity between the employed model system and that previously observed in different studies involving foods. Thus, when the water-holding capacity of meats was increased, heterocyclic aromatic amine formation was reduced (Persson *et al.*, 2003). In addition, extreme dehydration obtained with hot-air jets

resulted in low water activity and also resulted in a reduction of heterocyclic aromatic amines (Kondjoyan *et al.*, 2010).

According to the pathway shown in **Figure 92,** IQ formation requires one molecule of acrolein and one molecule of crotonaldehyde, and MeIQ formation requires two molecules of crotonaldehyde. Therefore, if both pathways were equally favoured, a similar amount of IQ and MeIQ should have been produced when 3.3 μmol of acrolein and 10 μmol of crotonaldehyde were heated in the model system. However, as shown in **Figure 65**, 25 μmol of acrolein and 10 μmol of crotonaldehyde were required to produce identical amounts of IQ and MeIQ, which suggested the involvement of acrolein in further reactions.

One of these side reactions of acrolein is the formation of MeIQx. A reaction pathway for the formation of MeIQx by reaction of acrolein and creatinine is proposed in **Figure 93**. Analogously to other heterocyclic aromatic amines, the reaction is proposed to be initiated by addition of creatinine (**2**) to acrolein (**15**) to produce the corresponding adduct (**16**). This adduct reacts then with ammonia, to produce the corresponding imine (**17**), which is in equilibrium with the enamine (**18**). The next step is the reaction of compound **18** with methylglyoxal (**24**), to produce the imine (**25**), which can react with a new molecule of ammonia to produce firstly the imine (**26**) and, then the amine (**27**) as a consequence of a tautomerism imine-enamine. This last compound has most atoms of MeIQx structure. Only one carbon atom is required to close the ring. Analogously to PhIP (Zamora *et al.*, 2014) this atom is suggested to come from formaldehyde (**28**). Thus, formaldehyde (**11**) would react with the amino group of compound **27** to produce the corresponding imine (**29**). Formation of MeIQx (**34**) from compound **29** is suggested to be produced by several steps that would include electronic rearrangements to produce firstly compound **30** and, then, compound **31**, followed by a dehydration to produce compound **32**, then a new electronic rearrangement to produce compound **33**, and, finally, an aromatisation to produce MeIQx (**34**).

As can be observed, only acrolein and creatinine are required to produce MeIQx. Thus, methylglyoxal (**24**) can be produced by oxidation of acrolein (**15**), ammonia is produced by thermal degradation of creatinine (**2**), and formaldehyde (**28**) is produced by degradation of acrolein (**15**). All the oxidations and degradation have been described previously (Zamora *et al.*, 2014; Zamora *et al.*, 2015b).

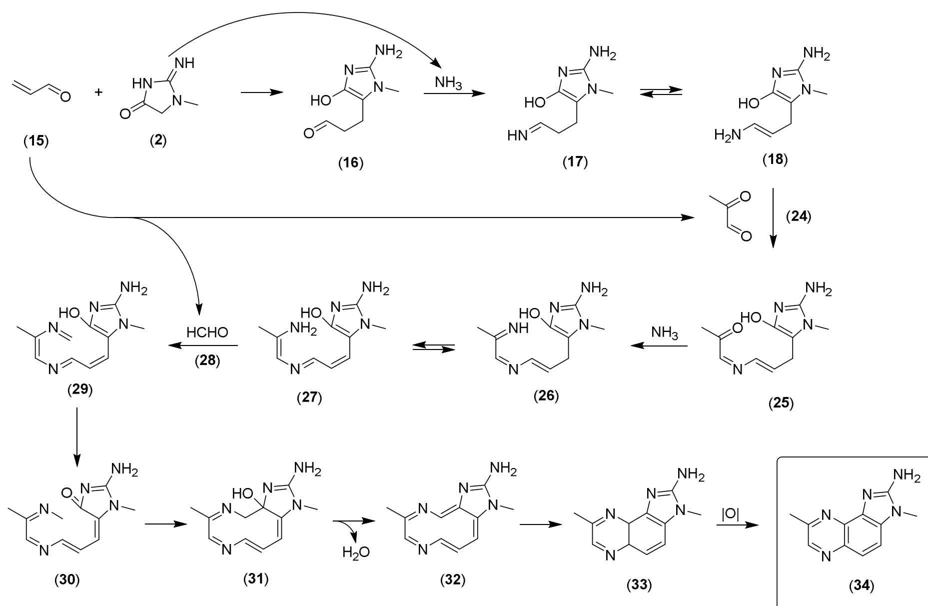


Figure 93. Proposed formation pathway for MeIQx.

According to described pathways, when others precursors with similar structures are present other similar heterocyclic aromatic amines should be produced. Thus, the reaction of 2-pentenal and crotonaldehyde in the presence of creatinine, and ammonia should produce 2-amino-3-ethylimidazo(4,5-*f*)-quinoline (EtIQ) by the pathway described in **Figure 91**. Moreover, the reaction pathway proposed in **Figure 93** should be general for the different heterocyclic aromatic amines with the structure of quinoxaline found in food products (Zamora & Hidalgo, 2015). Thus, for example, 4-MeIQx can be hypothesised to be produced in mixtures of crotonaldehyde and glyoxal with creatinine, and 4,8-

DiMeIQx can be hypothesised to be produced in mixtures of crotonaldehyde and methylglyoxal (or in mixtures of crotonaldehyde and acrolein) with creatinine.

5.4. Strategies for the mitigation of heterocyclic aromatic amines in foods

The above described identification of precursors and reaction pathways for the formation of heterocyclic aromatic amines provides evidences about both the origin of these food toxicants and the procedures by which the formation of these compounds can be inhibited. As discussed previously, heterocyclic aromatic amines formation mostly depends on the composition of the food carbonylome (the reactive carbonyls present/formed in a food that are continuously being produced and disappearing). This suggests that the most appropriate way to control the heterocyclic aromatic amine formation is to control formation and fate of this spectrum of reactive carbonyls. In this way, strategies should be focused to avoid reactive carbonyl formation (i.e., the inhibition of lipid oxidation or Maillard reaction), to employ reaction conditions that do not favour the conversion of reactive carbonyls into heterocyclic aromatic amines (i.e., the control of heating time and temperature), and to promote reactive carbonyl trapping (i.e., the use of phenolic compounds as carbonyl scavengers). In fact, the observed effectivity of phenolics for inhibiting heterocyclic aromatic amine formation are likely related to both, the well-known inhibition of lipid oxidation by phenolics (Muñoz-González *et al.*, 2019), and the ability of these compounds to trap the reactive carbonyls produced either as a consequence of Maillard reaction (Zhang *et al.*, 2019) or in the course of the lipid oxidation pathway (Hidalgo, *et al.*, 2018b; Hidalgo & Zamora, 2019).

6. CONCLUSIONS

First. The tendency of reactive carbonyls with the structure of 2-alkenals, 2,4-alkadienals, and 4,5-epoxy-2-alkenals, to evolve into heterocyclic derivatives in the presence of ammonia and ammonia-producing compounds has been described for the first time.

Second. This reaction plays a role in the sensory properties of foods, because of the formation of alkyl- and hydroxy-pyridines, and also in food safety, because it is responsible for the formation of carcinogenic heterocyclic aromatic amines with the structure of aminoimidazoazarene.

Third. Acetaldehyde/crotonaldehyde mixtures and 2,4-alkadienals are the main responsible for the formation of 2-alkylpyridines; acrolein and 2,4-alkadienals are needed for the formation of 3-alkylpyridines; and 2-alkenals are responsible for the formation of 2,5-dialkylpyridines. On the other hand, 2,6-dialkylpyridines are produced by cyclization of unsaturated ketones. Obtained results point out to lipid oxidation as the process responsible for the formation of these flavor compounds in food products.

Fourth. Reaction pathways for the formation of all these pyridines have been proposed and confirmed by using isotopic labeling experiments. All these pathways start with the addition of ammonia to the carbon-carbon double bond of the alkenal, and are followed by the formation of an imine with a second molecule of a reactive carbonyl, and the later cyclization and aromatization of the produced adduct.

Fifth. 3-Hydroxypyridines are produced by cyclization of 4,5-epoxy-2-alkenals in the presence of ammonia. Because 2,4-alkadienals are easily converted into 4,5-epoxy-2-alkenals under oxidative conditions, these pyridines are also easily produced from 2,4-alkadienals with an activation energy of 50 kJ/mol.

Sixth. A reaction pathway that explains the formation of 3-hydroxypyridines in the course of the lipid oxidation pathway has been proposed. It has been confirmed by studying the formation of these pyridines in oxidized linseed and menhaden oils, which were heated in the presence of glutamine as ammonia-producing compound.

Seventh. 3-Hydroxypyridines can also be produced in the course of Maillard reaction by ring expansion of the common Maillard products 2-oxofurans. Thus, 5-hydroxymethylfurfural (HMF), furfural, and 2-acetylfuran were converted into 2-hydroxymethyl-5-hydroxypyridine, 3-hydroxypyridine, and 3-hydroxy-2-methylpyridine, respectively, when heated in the presence of ammonia. A reaction pathway that explains this ring expansion has been proposed.

Eighth. The conversion of HMF into 2-hydroxymethyl-5-hydroxypyridine has an activation energy of 74 kJ/mol, which is higher than that determined for the formation of 3-hydroxypyridines from 2,4-alkadienals. Nevertheless, these reactions occurred in foods. Thus, when honey and sugarcane honey were heated the formation of 2-oxofurans and, also, of 3-hydroxypyridines was observed. The main 3-hydroxypyridine formed was 2-hydroxymethyl-5-hydroxypyridine.

Ninth. When creatinine was included in the reaction mixture, heterocyclic aromatic amines with the structure of aminoimidazoazarene were produced in addition to pyridines. Nevertheless, these compounds were always produced to a much lower extent than pyridines.

Tenth. The formation of three heterocyclic aromatic amines was studied: MeIQ was produced in mixtures of crotonaldehyde and creatinine with an activation energy of 72.2 kJ/mol; IQ was produced in mixtures of acrolein, crotonaldehyde, and creatinine with an activation energy of 77.0 kJ/mol; and MeIQx was produced in mixtures of acrolein and creatinine with an activation energy of 81.1 kJ/mol.

Eleventh. The formation of these heterocyclic aromatic amines also requires the presence of ammonia, which can be generated *in situ* by creatinine decomposition, and, in the case of MeIQx, also of methylglyoxal and formaldehyde, which are produced *in situ* by decomposition of acrolein.

Twelfth. Reaction pathways for the formation of all these heterocyclic aromatic amines have been proposed. All of them start with the addition of creatinine to the 2-alkenal. Then, ammonia produces an imine with the carbonyl carbon of the alkenal, and the produced adduct reacts with a second molecule of carbonyl compound. This process is finished with the stabilization of the produced compounds through the aromatization of the formed heterocyclic rings.

Thirteenth. All these results demonstrate the key role of reactive carbonyls (mainly acrolein and crotonaldehyde) on the formation of heterocyclic aromatic amines. Because creatinine is ubiquitous in proteinaceous foods, and ammonia-producing compounds are present in foods to a high extent, the control of the reactive carbonyls responsible for the formation of these heterocyclic aromatic amines seems to be the key point to limit the formation of these toxicants in food products.

Fourteenth. Strategies for limiting the formation of heterocyclic aromatic amines in foods should be focused in avoiding reactive carbonyl formation, employing reaction conditions that do not favor the conversion of reactive carbonyls into heterocyclic aromatic amines, and promoting the reactive carbonyl trapping.

Fifteenth. The observed effectiveness of phenolic compounds to inhibit heterocyclic aromatic amines in foods is likely a consequence of their combined ability for inhibiting the formation of reactive carbonyls and for scavenging the reactive carbonyls once they have been produced.

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