Pol. J. Food Nutr. Sci., 2013, Vol. 63, No. 4, pp. 207-225
DOI: 10.2478/v10222-012-0082-4
http://journal.pan.olsztyn.pl

Review article
Section: Food Chemistry

5-Hydroxymethyl-2-Furfural (HMF) – Heat-Induced Formation, Occurrence in Food and Biotransformation – a Review

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Key words: 5-hydroxymethyl-2-furfural (**HMF**); **HMF** synthesis; biotransformation; **HMF** metabolism; impact on health; heat indicator

The chemical structure of 5-hydroxymethyl-2-furfural (**HMF**), its physicochemical properties and reactions that lead to the synthesis of **HMF** were discussed. Special attention was paid to **HMF** formation in food during processing. The potential applications of this compound in industry were described as well. Moreover, this review outlines the most important sources of **HMF** in human diet and estimates the potential daily intake of **HMF** by consumers. The known and suggested metabolic pathways, as well as the impact of **HMF** and its metabolites on human health are also discussed.

INTRODUCTION

Food products are subjected to thermal treatment such as cooking, baking, roasting, extrusion cooking, pasteurization or sterilization, *etc.* These processes are commonly used to obtain desirable sensory or texture features, or to assure microbiological safety as well as to eliminate enzymatic activities. The reactions connected with thermal processing are very important for the production of sensory attributes like aroma, taste and colour. For some types of products including milk or fruit juices, these modifications must be reduced as much as possible in order to leave a natural, fresh appearance and taste as well as optimal nutrition value. However, in other cases they are desired, in order to produce the specific product quality. Good examples of such kind of products are: bakery goods, cereals, chocolate, coffee, nuts, malt and grilled meat. It is important to note that, when these thermal processes take place during food preservation, they always have an impact on quality. During thermal treatment of food products, the so-called Maillard Reaction or non-enzymatic browning occurs. These processes involve reactions of amino acids, peptides and proteins with reducing sugars and other carbonyl compounds. The chain of complex, competitive and consecutive reactions and the fact that they take place simultaneously generating many reactive intermediates make their interpretation and control difficult [Richardson, 2001]. Furan and many of its derivatives are one of the compound classes that can be found at very low levels in many foods and drinks as a result of thermal treatment [Vranová & Ciesarová, 2009]. The processes characterised by intensive thermal treatment are baking, toasting and roasting. In case of bread baking, time and temperature of the process are highly correlated with product dimensions. Small products (approx. 45 g) can be processed in about 20 min at 240–250°C, while larger ones (up to 1000 g) can be heated for up to 60 min or longer at 220°C. During this process, Maillard Reactions as well as caramelisation of sugars occurs, especially on the product's surface [Richardson, 2001].

The intensity of changes detected during thermal processing of food is usually monitored by the increase of concentrations of some indicators including 5-hydroxymethyl-2-furfural (**HMF**) or furosine [Rufían-Henares *et al.*, 2008]. **HMF** has especially been used for this purpose for a long time. On the other hand, **HMF** present in foodstuffs, seems to be studied extensively not only as a useful index of thermal processing but also as a food contaminant with potential harmful properties [Lee *et al.*, 1995; Hiramoto *et al.*, 1996; Albala-Hurtado *et al.*, 1998; Hidalgo & Pompei, 2000; Fallico *et al.*, 2003; Gökmen *et al.*, 2008; Husoy *et al.*, 2008].

HMF SYNTHESIS AND CHEMISTRY

5-Hydroxymethyl-2-furfural (CAS: 67–47–0) is a six-carbon heterocyclic aldehyde, a derivative of furan, containing both aldehyde and alcohol (hydroxymethyl) functional groups. The structure of the ring is based on furan moieties whereas the two functional groups, *i.e.* formyl and hydroxymethyl group, are linked with the ring at 2 and 5 positions, respectively (Figure 1).

All important physicochemical properties of **HMF** have been presented in Table 1.

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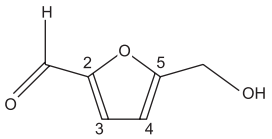

FIGURE 1. Structure of 5-hydroxymethyl-2-furfural **(HMF)**.

Due to its structure and occurrence in agricultural, carbohydrate-rich sources, **HMF** may be treated as a renewable material and can be an intermediate and/or a key molecule between the so-called "green chemistry" and mineral oil-based industrial organic chemistry. The main sources of **HMF** are different types of sugars – especially fructose [Kuster, 1990]. The substrate origin is application independent. **HMF** can be a product of thermal processing of fructose-rich food but it may also be a heavy-scale chemical. Of these products, 2,5-bis(hydroxymethyl)-furan (**BHMF**), 5-hydroxymethyl--2-furoic acid (**HMFA**), and the 2,5-dicarboxylic acid (**FDCA**) have already been extensively investigated for the preparation of furanoic polyesters. Some other **HMF** derivatives are also important from the application point of view. The group includes: [5-(aminomethyl)furan-2-yl]methanol (**AMFM**); furan-2,5-diyldimethanamine (**FDDMA**); furan-2,5-dicarbaldehyde (**FDCAL**); 5,5'-(oxydimethanediyl)difuran-2-carbaldehyde (**ODFCA**) among others. **HMF** may also play a role as an intermediate in levulinic acid (**LVA**) production. **LVA** is a monocarboxylic acid having a ketone carbonyl group at the δ-carbon position (4-oxopentanoic acid). **LVA** has many interesting chemical and physical properties that allow its use at different fields of application including: food and flavouring agents, as well as an intermediate for the preparation of a variety of industrial and pharmaceutical compounds, plasticizers, textiles, coating materials, and anti-freezers [Ghorpade & Hanna, 1999; Girisuta *et al.*, 2006; Corma *et al.*, 2007; Timokhin *et al.*, 2011].

HMF plays an important role in a biofuel production from different types of biomasses. Biomass pre-treatment by acid hydrolysis generates many growth inhibitor-like compounds, such as furfural (**F**) and **HMF**. The inhibitors reduce the growth of yeasts and bacteria and deteriorate subsequent alcoholic fermentation [Pfeifer *et al.*, 1984; Zaldivar *et al.*, 1999]. The development of furan-resistant biocatalysts could eliminate most problems experienced during the process. It has been demonstrated that several intestinal bacterial genera (*Klebsiella*, *Enterobacter*, *Escherichia*, *Citrobacter*, *Edwardsiella*, *Proteus*) convert **HMF** into 5-hydroxymethyl furfuryl alcohol, a less toxic compound [Boopathy *et al.*, 1993; Zaldivar *et al.*, 1999]. Recently, many new ethanologenic strains of the yeast *Saccharomyces cerevisiae*, resistant to **HMF** and **F**, were developed. These strains have the potential to transform **F** and **HMF** into, furfuryl alcohol and furan dimethanol (**FDM**), respectively [Liu *et al.*, 2005, 2008].

TABLE 1. Physicochemical properties of 5-hydroxymethyl-2-furfural [Haynes, 2010; Hoydonckx *et al*., 2007; O'Neil, 2006].

Properties	Value	Unit
Molecular formula	$C_6H_6O_3$	-
Molecular weight	126.11	g/mol
Boiling point	110 (at 2.67Pa); 114–116 (at 133.32 Pa)	°C
Melting point	31.5	°C
Density	1.2062	g/cm^3
Refractive Index	1.5627 (at 18°C)	-
Flash point	79	°C
logP (o/w)	-0.09	-

In general, **HMF** is a product of hexose transformation in the presence of acids at high temperatures. The mechanism of this reaction is fairly complex. When model compounds are used (Figure 2), *i.e.* there are no proteins or amino acids in the reaction media, the mechanism may be divided into two separate paths [Locas & Yaylayan, 2008]. One path includes the transformation of the fructofuranose ring, while the second one results in an acyclic intermediate.

The basic substrate for **HMF** production are monosaccharides *i.e.* fructose (**1a**) or glucose (**1b**) [Corma *et al.*, 2007]. Both of them may be used as a primary substrate or obtained in the process of oligo- or polysaccharide acid/enzymatic hydrolysis. Most of industrially important polysaccharides are built from anhydroglucose (starch, cellulose, *etc.*) or anhydrofructose (inulin, kestose, levan *etc.*) repeated units [Heinze *et al.*, 2005]. On the other hand, one of the most important disaccharides – sucrose, may be easily hydrolysed into (**1a**) and (**1b**) [Mathlouthi & Reiser, 1994]. As it is known the dehydratation of (**1a**) is more effective and faster than in case of (**1b**) [Locas & Yaylayan, 2004; Yaylayan, 2006; Zhao *et al.*, 2007]. Glucose as an aldohexose may be involved in competing reaction pathways leading to nonfuran cyclic ethers by-products formation. The C-C bond scissions may also occur in this case [Kabyemela *et al.*, 1997; Jing & Lu, 2008]. On the other hand some enhancement of **HMF** yields obtained from sucrose as a carbohydrate source was observed. It is the result of inversion of sucrose in acidic conditions. The main product of glycosidic bond cleavage in sucrose is fructofuranosyl oxocation (**3**), the direct precursor of **HMF** [Locas & Yaylayan, 2008].

The transformation of fructose (**1a**) to **HMF** occurs in three distinct dehydration steps which have been demonstrated using isotope labelling [Antal *et al.*, 1990]. The first stage of transformation involves dehydration of the furanose ring at C_5, with fructofuranosyl oxocation (**3**) being formed as a result. The elimination of a proton is the next step, resulting in the formation of a "vinyl alcohol-like derivative", the enol of 2,5-anhydro-D-mannose (**4**). Next, a water molecule is released from the enol closing the first double bond in the furan ring (**5**). The carbonyl group at C_5 of (**5**) arises at this stage. The third and last dehydration gives the final structure of 5-hydroxymethyl-2-furfural [Locas & Yaylayan, 2008]. In case of discussed mechanisms each of the steps was proved by means of isotope labelling investigation [Antal *et al.*, 1990]. The reaction carried out in D_2O as a solvent guided to the product (**HMF**) with no deuterium atoms present in the aldehyde structure.

When (**1b**) is present in the reaction medium, the mechanism is more complicated. First of all in acidic conditions (**1b**) may undergo Lobry de Bruyn–Alberda van Ekenstein transformation (Figure 3) [Milijkovic, 2009]. The reaction

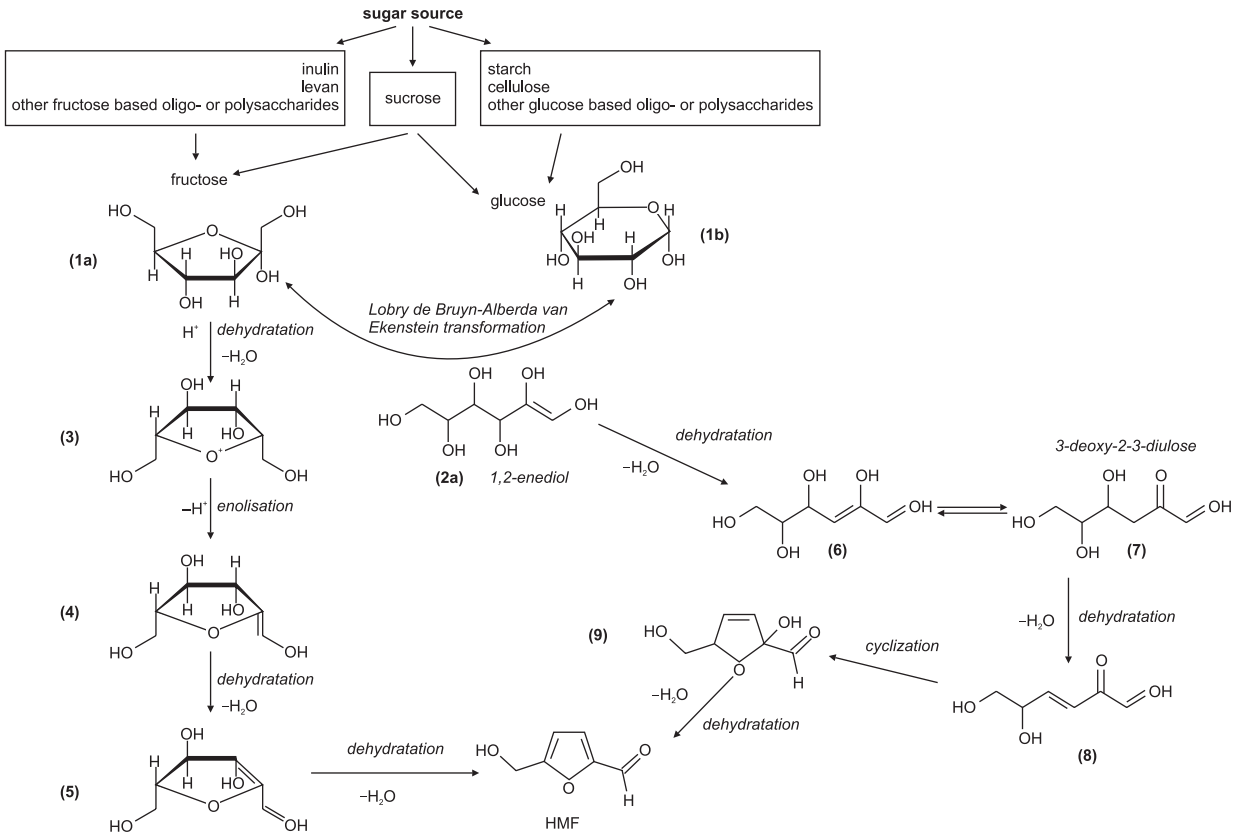

FIGURE 2. Model mechanism of **HMF** production from simple sugars.

results in (**1b**) isomerizing into (**1a**). The isomerization takes place with a tautomeric 1,2-enediol (**2a**) and 2,3-enediol (**2b**) as reaction intermediates. Both compounds: (**1a**) and the intermediate (**2a**) are employed in further steps of the process. Additionally, psicose (**1c**) and mannose (**1d**) may also appear as the result of Lobry de Bruyn–Alberda van Ekenstein transformation. The scheme presented (Figure 3) illustrates the broad range of monosaccharides involved in reaction mechanism as substrates. It is important to emphasize that most polysaccharides are acid-sensitive and may be hydrolysed into simple sugars, which subsequently act as starting material for **HMF** formation. Whilst

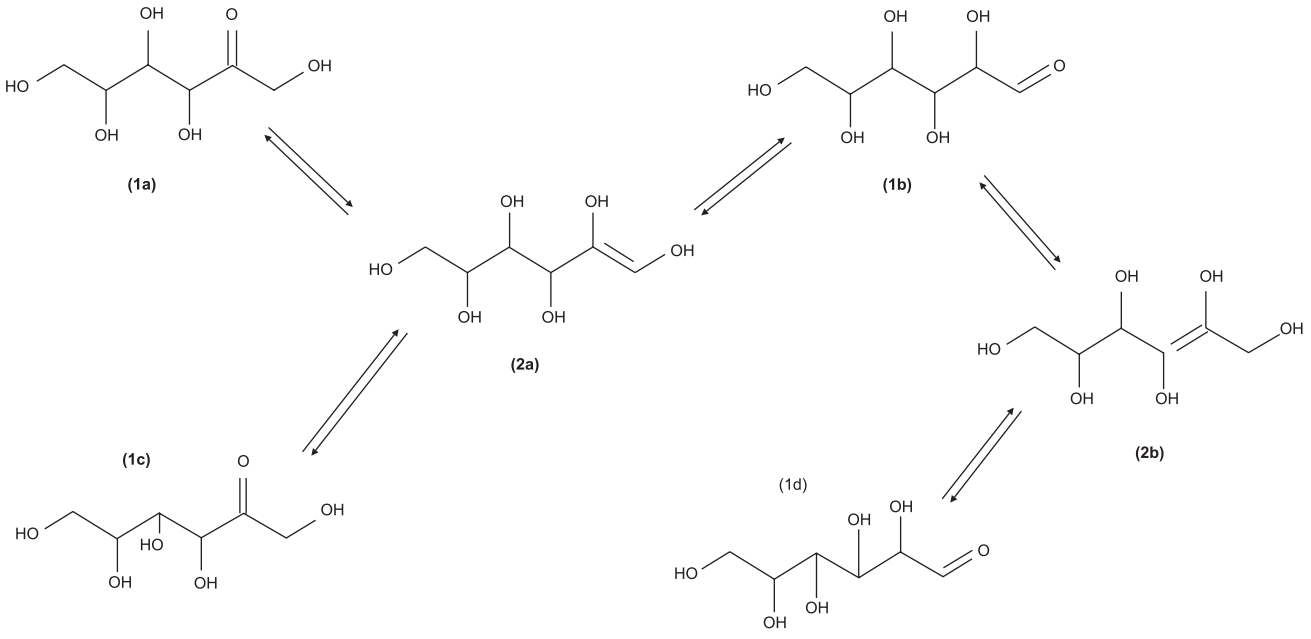

FIGURE 3. Lobry de Bruyn–Alberda van Ekenstein transformation.

acid hydrolysis is difficult to carry out, some enzymes may aid to bring polymers into monosaccharides that in acidic media, will isomerize into fructose (**1a**) or glucose (**1b**). After depolymerisation, according to the Lobry de Bruyn–Alberda van Ekenstein transformation, they may isomerize to form fructofuranose monosaccharides. Although fructose (**1a**) may be an initial substrate for cyclic mechanism path, the enediol is subjected to a dehydration step resulting in the closing of the C_3-C_4 double bound. The unsaturated hydroxyaldehyde (**6**) is a tautomeric form of 3-deoxy-2,3-diulose (**7**), an important intermediate for many side reactions. When no other compounds are present, (**7**) is subjected to the second dehydration step forming dicarbonyl compound with double bond at C_3 (**8**). The next stage is a cyclization resulting in 5-hydroxymethyl-2-formylo-2-hydroxy-2,5-dihydrofuran (**9**) that may lose a final water molecule resulting in 5-hydroxymethyl-2-furfural [Antal *et al.*, 1990].

Additional phenomena, strictly linked with **HMF** formation are many consecutive reactions of the final product that may occur even if only the sugar (substrate) and acid (catalyst) are present in the reaction medium.

In the case of **HMF** formation in food, various mechanisms should be taken into consideration. **HMF** is known as one of the important products when carbohydrate-rich food undergoes thermal treatment [Capuano & Fogliano, 2011]. Unfortunately, with the exception of sugar matrices there are also some other food ingredients present including fats, mineral compounds and proteins. Proteins and amino acids as a product of proteins hydrolysis take part in **HMF** formation in food [Fox, 1992; Friedman, 1996; Belitz *et al.*, 2009; Capuano & Fogliano, 2011] however, in this case two separate mechanisms may take place because fructose (**1a**) and glucose (**1b**) will give the same product (**HMF**) through different intermediates (Figures 4, 5) [Yaylayan *et al.*, 1994; Yaylayan & Ismail, 1995; Yaylayan, 1997; Locas & Yaylayan, 2004].

In case of glucose (**1b**), the very first stage is the reaction with the amino group (NH_2-) of the amino acid or protein (Figure 4). As a result a Schiff base (**10**) is produced which is a tautomeric form of N-substituted glucosylamine (**11**). The compound then undergoes protonation to a Schiff base cation (**12**). After the protonation followed by a ring opening, the Schiff base may be enolised and deprotonated giving appropriate 1,2-enaminol (**13**). At this stage two mechanistic paths are possible. One of them is the rearrangement of (**13**) into N-substituted 1-amino-1-deoxy-2-ketose (**14**). Further transformation of (**14**) *via* the cyclization step results in the so-called Amadori compound (**15**). The Amadori product has various keto-enol tautomers that may undergo deamination, dehydration, fragmentation or enolisation. It gives a large collection of follow-up products containing different amounts of carbonyl groups including furan (furfural, furanones) or pyran (pyranones) derivatives. Some low molecular compounds may also be produced at this stage, such as formic acid which may serve as an autocatalyst for some acid-dependent dehydration steps [Yaylayan *et al.*, 1994; Miljkovic, 2009].

On the other hand, in acidic media (**13**) may lose a water molecule forming eneiminol (**16**). Deamination of (**16**) with

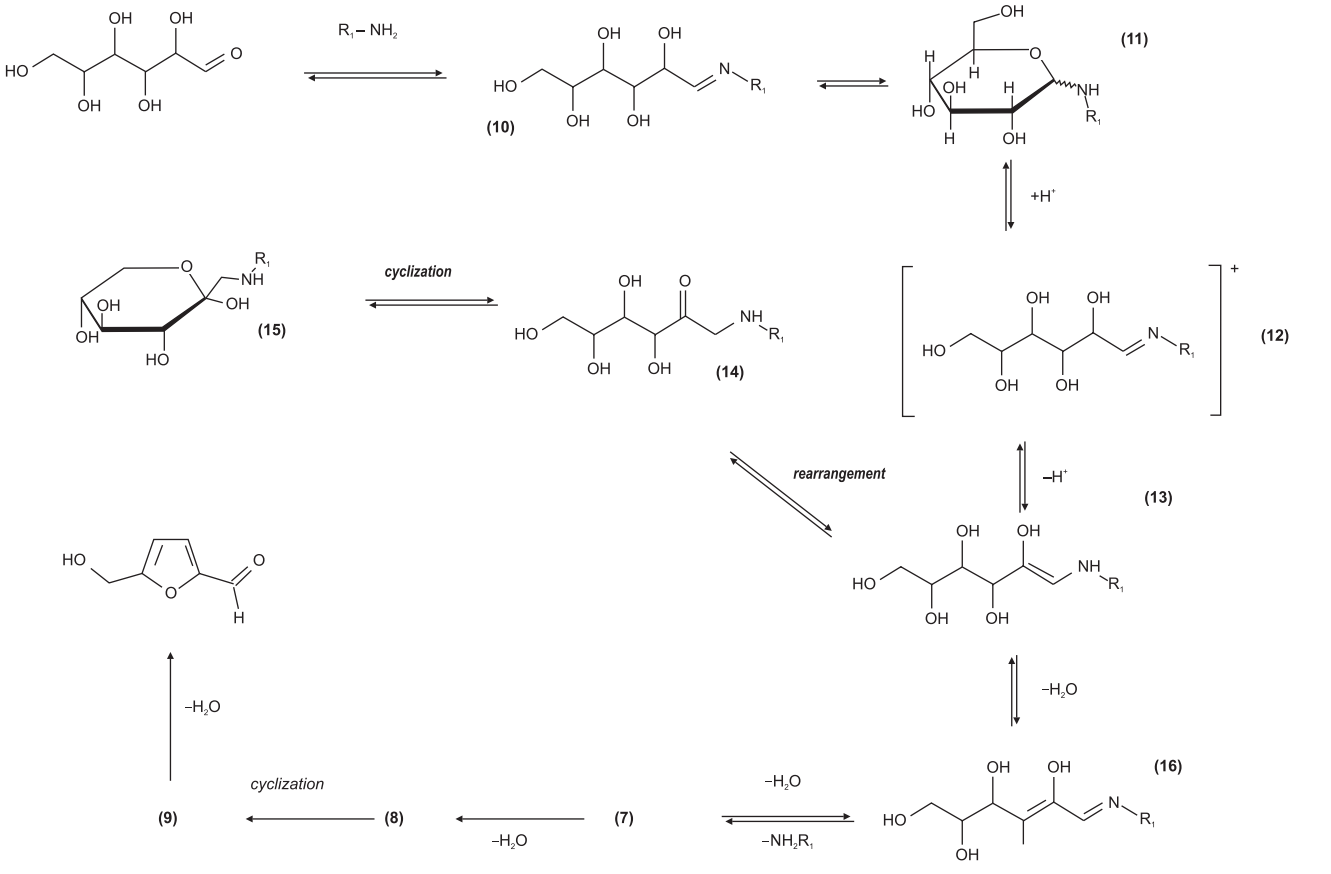

FIGURE 4. Transformation of glucose to **HMF** in food.

subsequent addition of water results in the formation of 3-deoxy-2,3-diulose (**7**). Further transformations of (**7**) are similar to those described earlier (Figure 2) and leads to **HMF** *via* (**8**) and (**9**).

When **HMF** synthesis starts with fructofuranose (**1a**) the mechanism may look similar, although this is true for deeper stages of the reaction. The initial steps are fairly different in this case (Figure 5) [Brands & van Boekel, 2001, 2003; Miljkovic, 2009]. (**1a**) may be present in the reaction mixture as both cyclic and chain form. Only the second form may be involved in the reactions with amino groups. In this case, the carbonyl group is placed at C_2 position employing the ketone group of the sugar. As a result an additional compound (**17a**) arises that can easily lose the water to give a Shiff base (**17b**) that is in equilibrium with its cyclic form *i.e.* N-substituted ketosamine (**17c**). Ketosamine may be protonated by acids resulting in ring opening (**18**). Further protonation forms enaminol (**19**) that may rearrange to 2-amino-2-deoxyaldose (**20**). Cyclization of (**20**) gives the so-called Heyns product. The Heyns product is a counterpart of the Amadori compound observed for compounds possessing aldehyde group, such as (**1b**). Alternatively (**20**) may be transformed into an **HMF** moiety *via* dehydration and the second amination stages. In this case, (**20**) will first be aminated resulting in a diamine production that may be presented in several tautomeric forms including 1-amino-2-imino compound. Deamination and hydration of this compound result in 1-amino-1-deoxy-2-ketose (**14**). The mechanism may be treated as an interconversion of Heyns and Amadori compound. Due to this transformation, both mechanisms (Figures 4 and 5) could be involved in **HMF** formation and the process can be treated as sugar type independent. The compound (**14**) is employed in further transformations *via* (**13**) and (**16**) and finally, as it was described earlier by steps (**7**) – (**9**), will give **HMF** as a final product.

As it was shown, **HMF** may be synthesised if food or food products, that are rich in carbohydrates and/or amino acids, are subjected to any thermal processing. Recently it has also been shown that some substances presented in food may work as catalysts that enhance **HMF** and other furan aldehyde concentrations in food products. Preliminary studies in this field clearly show that in model systems multivalent metal cations including calcium and magnesium increase the yield of **HMF** and **F** [Gökmen & Şenyuva, 2007]. In turn, in case of industrial production of **HMF** as a starting compound for chemical industry various kinds of both Broenstaed and Lewis acids have been employed to increase the **HMF** content. According to Lewkowski [2001], there are hundreds of different acids or salts able to catalyse the dehydratation of sugars [Lewkowski, 2001]. The search for new ones is the simple consequence of reactivity of a desired product – **HMF** that is rather unstable compound, and subsequent reaction including polymerization of **HMF** may occur [Ashry, 2007].

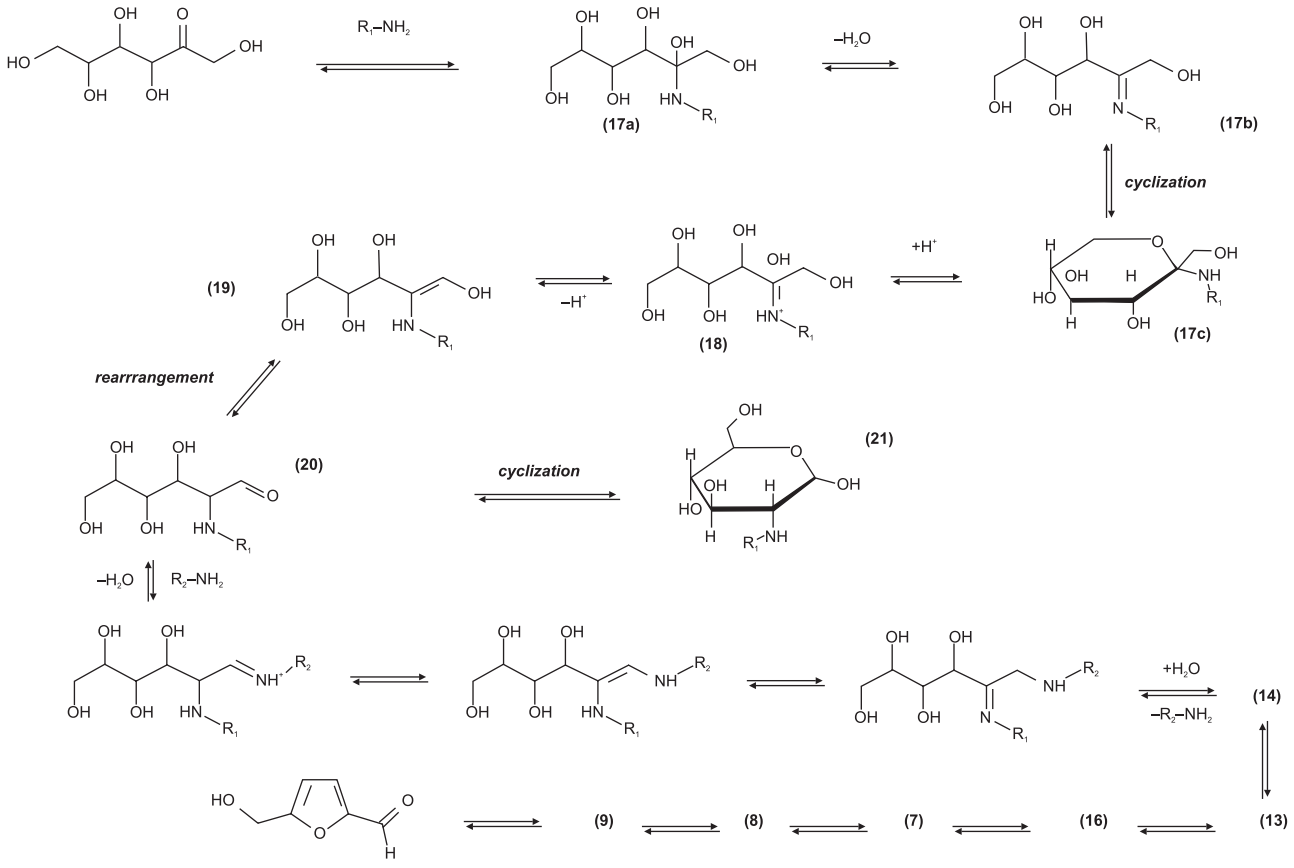

FIGURE 5. Transformation of fructose to **HMF** in food.

HMF IN FOOD AND RELATED PRODUCTS

The previously discussed mechanisms and favourable conditions for the formation of **HMF** are frequently encountered in the food industry. Thermal processing (roasting, toasting baking, sterilization *etc.*) especially of carbohydrate-rich foodstuffs has a huge impact on the process. Particularly breakfast cereals, coffee, bread as well as pasteurized juices or pulps *etc.* are subjected to intensive **HMF** formation.

Cereal products

Rufían-Henares *et al.* [2006] investigated **HMF**, **F** as well as glucosylisomaltol contents as indicators of thermal processing intensity of breakfast cereals. Analysis of sixty commercial available products from Europe and the USA revealed that the **HMF** concentration varied between 6.59 and 240.51 mg/kg (w/w). Nevertheless, values above 100 mg/kg were found in just five cases. According to this work, the highest average concentration of **HMF** was found in maize-based breakfast cereal (42.81±7.92 mg/kg), followed by wheat (40.79±8.57 mg/kg) and rice (32.14±10.79 mg/kg) products. The lowest **HMF** content was determined in mixed cereal flakes (26.79±11.59 mg/kg). Authors have also compared products with and without the addition of honey and stated that **HMF** concentration was higher in the former group, 43.44±10.35 *versus* 34.24±6.17 mg/kg, respectively. In the case of cereals supplemented with cocoa, **HMF** concentration was lower (28.68±12.8 mg/kg) than without this addition (39.48±5.31 mg/kg). However, there were no statistically significant differences between the analysed groups regardless of raw materials used and additives applied [Rufían-Henares *et al.*, 2006].

Ramírez-Jiménez *et al.* [2003] investigated non-enzymatic browning of infant rice-based cereals during storage at different temperatures (25, 32, 55°C) in the presence of air or nitrogen atmosphere with or without constant water activity (a_w). **HMF** concentration was measured as a browning indicator. It was stated that only at constant water activity (a_w=0.65) at a temperature of 25°C **HMF** formation was not observed. At higher temperatures, **HMF** concentration increased slightly and this phenomenon was more noticeable under nitrogen rather than air atmosphere (up to 2.3 and 1.86 mg/kg, respectively; initial **HMF** concentration 0.71 mg/kg; temp 55°C) [Ramírez-Jiménez *et al.*, 2003].

Nine types of bread commonly encountered in the Spanish market were analysed by Ramírez-Jiménez *et al.* [2000]. In this group, such products as baked or fried confectionery or wheat sourdough bread were investigated. The highest **HMF** concentration was detected in wheat bread (151.2 mg/kg), however, other breads from this group were characterised by much lower **HMF** content (approx. 40.5 mg/kg). According to the authors, this difference could be due to the presence of an egg layer coating on bread surface, which causes an increase in **HMF** content. This results from the presence of proteins that contribute to the synthesis of **HMF** in accordance with the previously discussed mechanisms. In case of bread, a higher concentration of **HMF** may be correlated with crumb and crust ratio. Bread with a thinner crust had a lower **HMF** concentration (4.1 mg/kg) than the thicker one (14.2 mg/kg). Confectionery products (doughnuts and croissant) had lower **HMF** concentrations (9.5 mg/kg) than biscuits (15.6 mg/kg) [Ramírez-Jiménez *et al.*, 2000].

In another study, Ramírez-Jiménez *et al.* [2000] examined six varieties of so-called "common" breads as well as nine varieties of "special" breads for **HMF** concentration and its influence on the browning index. For "common" breads, the **HMF** content varied between 3.4 and 68.8 mg/kg and correlated with water content in the product. Double fermentation of dough resulted in the highest **HMF** concentrations being observed in the final product. In the "special" bread group, the lowest **HMF** concentration was found in oat bread (4.8 mg/kg) and the highest in white bread containing dried fruits (51.3 mg/kg). In all cases large differences were found in **HMF** concentration between the crust and crumb. For white bread with the longest baking time values were 1.7 and 176.1 mg **HMF**/kg for crumb and crust, respectively [Ramirez-Jimenez *et al.*, 2000].

The influence of sugars (glucose, fructose and saccharose) content as well as water activity (a_w) and baking temperature on **HMF** formation in cookies was studied by Ameur *et al.* [2007]. It has been stated that **HMF** formation depends on baking temperatures (oven temperature 200; 250; 300°C) as well as water activity. Cookies baked at 200°C contained 10 to 100 times less **HMF** (9.9–39.6 mg/kg) than cookies baked at higher temperatures (167.4–1100.1 mg/kg). Up to 250°C, the cookies containing saccharose had lower levels of **HMF** (9.9 mg/kg) than those containing glucose or fructose (39.6––34.2 mg/kg). In products baked at the highest temperature (300°C), these authors observed a rapid increase in **HMF** content (1100.1 mg/kg) for cookies sweetened with saccharose. This phenomenon was associated with thermal degradation of saccharose [Ameur *et al.*, 2007].

Observations made by Ameur *et al.* [2007] were confirmed by Gökmen *et al.* [2007]. They studied the influence of raw material composition on **HMF** and acrylamide formation in cookies. The authors observed an increase in **HMF** concentration with increasing temperature of baking (from 160 to 230°C). This phenomenon was especially easy to observe in the case of glucose-containing cookies (≈36 mg/kg). Authors have also observed a rapid increase in **HMF** concentration as a function of pH decrease from neutral to acidic conditions. It was more evident in the case of cookies with saccharose than with glucose (up to 220 mg/kg and 30 mg/kg, respectively). According to authors the phenomenon may be result from disaccharide hydrolysis and fructose release into the reaction medium [Gökmen *et al.*, 2007]. In a consecutive work, Gökmen *et al.* [2008] focused on the influence of leavening agents, like sodium or ammonium bicarbonate, as well as sugars on **HMF** formation during baking of confectionary products. Like their predecessors [Ameur *et al.*, 2007], the authors recognised the significance of water activity in **HMF** formation. They also acknowledged that there was critical value of a_w (<0.4) below which a sudden increase in the rate of **HMF** synthesis was detected. Addition of ammonium bicarbonate resulted in an unexpected increase in **HMF** concentration just after 15 min of baking cookies containing saccharose (above 3500 mg/kg at 220°C). In the case of cookies containing glucose this increase was not as significant (up to 2000 mg/kg at 220°C).

Replacing ammonium bicarbonate with sodium bicarbonate generated a more or less twenty fold decrease in **HMF** concentration in cookies with saccharose and about two fold decrease when glucose had been used as a sweetening agent [Gökmen *et al.*, 2008]. The authors associated this phenomenon with the influence of leavening agents on pH changes. The use of ammonium bicarbonate was associated with a decrease in pH (especially at higher temperatures), which contributed to the decomposition of sucrose, and consequent increases in **HMF** concentration.

HMF as well as acrylamide was also analysed in *churros*, a traditional Spanish snack product. *Churros* are made by deep frying of dough pastry. It was found that under typical conditions for *churros* preparation (between 190 and 200°C) **HMF** concentration rapidly increased up to approximately 230 mg/kg [Morales & Arribas-Lorenzo, 2008]. On the other hand, Delgado-Andrade *et al.* [2010] have estimated an **HMF** level in *churros* at 19.5 mg/kg.

Kukurova *et al.* [2009] investigated the impact of asparaginase amount on the formation of acrylamide in *rosquillas* (Spanish donuts). Asparaginase was used at two concentrations (100 and 500 U/kg of flour respectively). Regardless of enzyme content present, a reduction of acrylamide up to 90% was observed in the final product, but no important influence on the **HMF** content was detected. The only observation in this case was a negligible increase in **HMF** concentration [Kukurova *et al.,* 2009].

HMF formation was also studied as one of the factors influencing browning of infant cereals. Increases in **HMF** concentration were investigated at different stages of cereals processing (toasting, hydrolysis, drying) in model systems [Fernandez-Artigas *et al.*, 1999]. Regardless of flour type, the hydrolysis process was connected with increases in **HMF** concentration. The drying stage, however, did not contribute to overall **HMF** synthesis probably due to short processing times. It was stated that in a model system containing both sugars and amino acids **HMF** was produced on a larger scale than in the sugar model system [Fernandez-Artigas *et al.*, 1999].

The impact of flour type (wheat, rye, whole-wheat) as well as toasting temperature (140, 160, 180°C) on **HMF** as well as acrylamide formation in toasted bread has also been studied [Capuano *et al.*, 2009]. The highest amounts of **HMF** were detected in rye and wheat breads toasted at 180°C (46.69 and 47.02 mg/kg, respectively). The lowest values were obtained in the case of whole-wheat toasts (29.22 mg/kg). After 34 min of toasting at 160°C, the authors determined 18.52, 14.63, and 7.86 mg **HMF**/kg of toasted bread (rye, wheat, whole-wheat, respectively). Toasting at 140°C for 40 min did not promote formation of significant amounts of **HMF**; 2.53 and 2.65 mg/kg for wheat and whole-wheat flours, respectively, and 4.48 mg/kg for rye flour bread. Glycine addition had a positive impact on increased **HMF** formation regardless of flour type used. Conversely, glycine reduced acrylamide formation in toasted bread. A higher level of **HMF** in toasted breads from rye flour was associated with a higher concentration of free amino acids and proteins [Capuano *et al.*, 2009].

In order to estimate the influence of flour type on **HMF** formation in cereal products, Rufían-Henares *et al.* [2009] analysed the toasting process in a model system. The authors suggested that high amounts of **HMF** in cereal products might be connected with ingredients other than flour. Untoasted flours had an initial level of 3 mg/kg of **HMF**, excluding rice flour (<0.025 mg/kg). The toasting of different flours (wheat, whole-wheat, corn, oat, rice, soybean) increased the **HMF** level up to 4.16, 4.72, 6.23, 3.43, and 12.53 mg/kg, respectively, except for rice flour (no change) [Rufían-Henares *et al.*, 2009].

Coffee

On the basis of analysis of twenty two coffee samples Murkovic & Pichler [2006] stated that **HMF** concentration in the investigated products ranged from 300 to 1900 mg/kg. Authors also focused on **HMF** residue in urine extracted within six hours after uptake of food samples with known **HMF** concentrations. Only 0.75% of ingested **HMF** was found unmetabolised in the urine extracts [Murkovic & Pichler, 2006]. In a follow-up study, Murkovic & Bornik [2007] investigated the formation of **HMF** as well as **HMFA** during coffee processing. They found that roasting coffee at 240°C caused a rapid increases in **HMF** (up to 900 mg/kg) in the first 3 min. In the case of **HMFA**, the maximum concentration of 150 mg/kg was determined after 4 min of processing. Further roasting was connected with decreases in **HMF** and **HMFA** contents probably because of the occurrence of consequent degradation reactions. Kinetic studies and model systems applied in research allowed to state that **HMFA** was formed from precursors other than **HMF**, namely pyruvate and glyceraldehyde [Murkovic & Bornik, 2007]. Arribas-Lorenzo & Morales [2010] analysed thirty five commercial roasted coffee brands from twenty one producers as well as nineteen soluble coffee brands from eleven producers. They estimated four levels of **HMF**: 110, 625, 1734, and 2480 mg/kg for natural, blend (mixture of torrefacto and natural coffee), torrefacto (coffee roasted with sugar addition) and soluble coffee, respectively. The largest differentiation in **HMF** level was found in soluble coffee clusters (min. 691, max. 4023 mg/kg). According to del Campo *et al.* [2010], **HMF** concentration in soluble coffee can reach 6180 mg/kg. In other groups analysed by Arribas-Lorenzo & Morales [2010] diversity was much lower for natural coffee (24–128 mg/kg), blends (303–1071 mg/kg) and torrefacto (1168–2186 mg/kg). The authors discussed the influence of different modes of coffee brewing (espresso, filtered, Italian, soluble) as well as Spanish consumer consumption habits on potential daily **HMF** intake. Obtained results allowed the authors to conclude that average daily coffee consumption resulted in **HMF** intake of about 5.26 mg/kg. It means that 75.15 μg **HMF** was received for 1 kg of body weight (adult average body weight 70 kg). For persons with high consumption habits a daily **HMF** intake was estimated at 8.57 mg/kg resulting in 122.42 μg **HMF**/kg body weight [Arribas-Lorenzo & Morales, 2010].

Fruit and vegetable products

Fruits and vegetables are usually rich sources of sugars and organic acids as well as amino acids. Processing of this group of foodstuffs thus leads to the formation of significant amounts of **HMF**. Rada-Mendoza *et al.* [2004] investigated two different jam products obtained in laboratory condi-

tions, one commercial product and one pear-banana infant dessert. The jam samples were stored for twelve months at 20 and 35°C. The impact of storage time on **HMF** formation was temperature dependent. Infant dessert had the lowest **HMF** concentration at both 20 and 35°C *i.e.* 6 and 65 mg/kg, respectively. Despite an initially higher content of **HMF** in the laboratory jam compared to the commercial one, the latter proved to be richer in **HMF** after storage. On the basis of those results, the authors confirmed the usefulness of **HMF** both as a heat processing index and an indicator of storage conditions [Rada-Mendoza *et al.*, 2004].

Murkovic & Pichler [2006] analysed several dried fruits including apricots, pears, peaches plums, dates, figs, as well as pineapples and apples. The highest concentrations of **HMF** were found in plums and dates (1100–2200 and 1000 mg/kg, respectively). In other samples, **HMF** concentrations ranged from 1 to 780 mg/kg [Murkovic & Pichler, 2006].

An increase in **HMF** concentration in vegetable products (bottled tomato puree) during 180 days of storage at 20°C was investigated by Ordóñez-Santos *et al.* [2009]. The increase from 3.95 up to 9.94 mg/kg was correlated with a decrease in organic acids, such as citric, ascorbic and malic acids [Ordóñez-Santos *et al.*, 2009].

Ibarz *et al.* [1999] elaborated a kinetic model of colour changes during thermal processing of pear puree between 80 and 98°C as well as a kinetic model of **HMF** formation. The authors considered two model types: second-order autocatalytic and first-order kinetics. In the first case, the rate constant was $0.95 \cdot 10^{-3}$ min^{-1} (80°C) and $6.27 \cdot 10^{-3}$ min^{-1} (98°C), in the second model $1.03 \cdot 10^{-3}$ min^{-1} (80°C) and $5.45 \cdot 10^{-3}$ min^{-1} (98°C) [Ibarz *et al.*, 1999].

Burdurlu & Karadeniz [2003] investigated the influence of extract, storage time and temperature on non-enzymatic browning of apple juice concentrates from two varieties of apples (*Golden Delicious* and *Amasya*). The obtained results (browning index measured by means of absorbance at 420 nm and *lightness* (L*) according to a CIE-Lab colour system) were correlated with **HMF** concentration. Juice samples of 65, 70 and 75°Bx were stored at different temperatures (5, 20, 37°C) for four months. For juices stored at 5°C as well as 20°C, increases in **HMF** level were minor (increase from 0.62 up to 4.37 mg/kg depending on variety, extract and temperature). **HMF** formation was much more significant at 37°C reaching 963 and 190 mg/kg for the *Golden Delicious* and *Amasya*, respectively. Results of **HMF** concentration in *Golden Delicious* juices stored at 37°C had good correlation with the browning index (r from 0.974 to 0.992). On the other hand, for juices stored at 20°C better correlation was found with lightness (L*) (r from -0.905 to -0.926). In the case of *Amasya* juice, a correlation coefficient above 0.9 was obtained only for results from juice storage at 37°C [Burdurlu & Karadeniz, 2003].

Analysis of **HMF** was employed to monitor pasteurization progress (microwave as well as conventional methods) of apple cider [Gentry & Roberts, 2004]. The authors determined rate constants of **HMF** synthesis in a model system (pH 3.8; 10°Bx) at temperatures between 25 and 80°C with asparagine addition (5 or 10 mmol/L). It was concluded that **HMF** formation occurred probably accordingly to an apparent zero-order reaction, with activation energy of 27.3 kJ/mol. The pasteurization process (needed for a 5-log reduction of *Escherichia coli* in apple cider) was associated with a calculated formation of 1.56 and 1.19 mg/kg of **HMF** in the case of microwave and conventional pasteurization, respectively. Those model system results were well correlated with pasteurization of real apple cider, where 1.57 and 1.2 mg/kg of **HMF** was obtained for microwave and conventional pasteurization, respectively [Gentry & Roberts, 2004].

Kinetics of **HMF** formation was also investigated in tomato products [Hidalgo & Pompei, 2000]. **HMF** formation followed apparent zero-order kinetics. Activation energy was estimated at 139.9 kJ/mol and z value (increase in temperature that causes a tenfold increase in the reaction rate) was 19.2°C. The authors revealed that in tomato pastes, degradation of **HMF** can occur, which clearly shows that **HMF** content cannot be used as a thermal processing indicator therein [Hidalgo & Pompei, 2000].

Wang *et al.* [2006] conducted research on non-enzymatic browning of carrot juice concentrate. Among the tested storage temperatures (-18, 0, 25, and 37°C), non-enzymatic browning took place mainly at two highest temperatures. It was stated that the degree of browning was in strong correlation with **HMF** formation and followed first-order reaction kinetics. The rate of **HMF** synthesis depended on temperature as well as juice extract concentration. For example, k rate constant for juices of 20 and 60°Brix at 25°C were 0.0119 and 0.0173 (1/day), respectively [Wang *et al.*, 2006].

Different factors, including sugar concentration, water activity and pH, were studied to estimate their influence on **HMF** formation in grape must [Muratore *et al.*, 2006]. Sugar concentration was controlled by cryoconcentration and water activity by NaCl addition. The influence of pH was studied in model solutions. It was established that juice extract and water activity had the greatest impact on **HMF** formation. The influence of low pH was also important but in real samples it could play a less important role (except heat processing where acids concentrations increases) [Muratore *et al.*, 2006].

Fallico *et al.* [2003] investigated the influence of oil concentration on **HMF** formation as well as on colour changes during roasting of hazelnuts. For this purpose hazelnut samples were defatted, ground, and subsequently roasted with varying amounts of hazelnut oil or oil containing hexanal and/or saccharose. Increased oil concentration resulted in increased browning intensity as well as increased **HMF** concentration. For non-defatted samples, prolonged roasting time (from 30 to 60 min) caused subsequent increases in **HMF** concentration (from 66.5 up to 144 mg/kg). The highest **HMF** concentration was observed in non-defatted hazelnuts with saccharose (372 mg/kg) in contrast to defatted hazelnut sample with saccharose (33.5 mg/kg) [Fallico *et al.*, 2003].

Several dehydrated vegetable samples were also analysed for **HMF** and furosine level as indicators of thermal treatment [Rufían-Henares *et al.*, 2008]. However, **HMF** was not detected in most cases excluding dehydrated vegetable extracts such as artichoke, tomato and cabbage (6.97, 18.2, and 58.6 mg/kg, respectively) [Rufían-Henares *et al.*, 2008].

Honey and confectionary products

In case of honey, the level of **HMF** is strictly normalized [Council Directive, 2001] and its analysis is commonly performed in many laboratories. According to normalization, **HMF** concentration in honey should not exceed 40 mg/kg with exception of honeys from tropical climate (not more than 80 mg/kg). Increased amounts of **HMF** in honey can result from improper processing or prolonged storage [Sancho *et al.*, 1992; Tosi *et al.*, 2002, 2004, 2008; Sanz *et al.*, 2003; Fallico *et al.*, 2004; Gidamis *et al.*, 2004; Zappala *et al.*, 2005; Nanda *et al.*, 2006; Spano *et al.*, 2006; Escriche *et al.*, 2008; Turhan *et al.*, 2008]. Sancho *et al.* [1992] investigated the influence of honey storage time on changes in quality parameters like enzymatic activity and **HMF** content. On the basis of 115 honey samples, the authors concluded that there was a logarithmic dependence between **HMF** concentration in honey and its storage time. They suggested establishing a freshness period for honey of two years after packaging, taking into consideration that in the second year of storage, quality deterioration is much faster than in the first one. For unpasteurized honey from Basque Country they suggested an **HMF** limit of 15 mg/kg just after honey harvest, blending and packaging. In the second year of storage the **HMF** level should not exceed 30 mg/kg [Sancho *et al.*, 1992]. On the other hand, Gidamis *et al.* [2004] focusing on selected Tanzania honeys, determined that honey should not be stored longer than 6 months in order to prevent unreasonable **HMF** increase [Gidamis *et al.*, 2004].

An ncreased **HMF** level in honey was also found to be connected with initial pH (acidity) [Fallico *et al.*, 2004]. Although the concentration of **HMF** in honey increased due to the heating process, this phenomenon did not reflect for how long and at what temperature the honey was heated. It was estimated that nectar honey processed at 95°C for 90 min as well as honeydew honey heated at 90°C for 75 min had **HMF** levels below 40 mg/kg [Turhan *et al.*, 2008]. Similar conclusions were drawn by other researchers [Tosi *et al.*, 2002, 2004, 2008]. They investigated the kinetics of **HMF** formation and changes in enzymatic activity during honey heating. It was shown that the initial **HMF** concentration did not influence the kinetics of its formation. It was also confirmed that even after intensive heating (90°C for 20 min) **HMF** concentration did not reach 40 mg/kg [Tosi *et al.*, 2002, 2004, 2008]. Escriche *et al.* [2008] stored honey samples from Spain at different temperatures (35–65°C). The resultant **HMF** concentration changes depended on temperature and storage time. For example, after 28 days of storage at 35 and 65°C **HMF** concentration increased up to 50 mg/kg and 240 mg/kg, respectively [Escriche *et al.*, 2008]. Khalil *et al.* [2010] found that after two years of storage, **HMF** amount in honey raised up to 1344 mg/kg from an initial value of 12.19 mg/kg.

Fallico *et al.* [2008] however, pointed out that under different storage conditions degradation of **HMF** can occur in honey samples. The estimated value of activation energy for the **HMF** degradation process was almost half the value of **HMF** formation energy regardless of the botanical origin of honey. Rate constants of degradation process at temperatures between 25 and 50°C (for citric as well as chestnut honey) were higher than the corresponding rate constant of formation. These findings should be taken under consideration in proper legislation process [Fallico *et al.*, 2008].

The impact of microwave processing on **HMF** content in different honey samples was investigated by Bartákova *et al.* [2011]. **HMF** concentration ranged widely among samples. It was found that it can even decrease during microwave processing. This indicated that this quality factor was unsuitable for use as an indicator of the heating process (or honey overheating) [Bartákova *et al.*, 2011].

Honey is commonly used for *turron* (typical Spanish confectionery) production. Regardless of honey origin and processing time, **HMF** appeared in *turron* but its content did not exceed 30 mg/kg. It was found that the **HMF** level in *turron* was strictly dependent on the initial concentration in honey [Vázquez *et al.*, 2007].

Dairy products

Sterilization processes are the origin of **HMF** in dairy products and may be connected with their colour change (browning). Albala-Hurtado *et al.* [1998] studied changes of **HMF** concentration during storage of infant milk. The authors stored samples for up to nine months at different temperatures (20, 30, 37°C). Free and total **HMF** (free **HMF** compounds plus the potential **HMF** compounds derived from other browning intermediates by heating sample with oxalic acid at 100°C for 25 min) were analysed. Powdered infant milk had more **HMF** present than corresponding liquid milks (34.7 and 12.2 μg/kg (w/v), respectively, after 9 month, 37°C). In this case, zero-order kinetics of **HMF** formation were established regardless of milk type and storage temperature [Albala-Hurtado *et al.*, 1998].

In traditional Indian dairy products (*Dudh churpi*), **HMF** levels are highly correlated with the sensory attributes of the product. A strong positive correlation was found between **HMF** content and colour, texture, flavour as well as overall appearance [Aktar Hossain *et al.*, 1999]. **HMF** concentration was also measured in several infant milk-based formulas [Morales & Jiménez-Pérez, 2001]. In most cases, the mean concentration of **HMF** was 29.5 μg/kg (w/v). In two samples it was found to be 296.6 and 247.2 μg/kg (w/v) [Morales & Jiménez-Pérez, 2001].

The influence of different temperatures on **HMF** formation during the storage of UHT milk was studied by Cais-Sokolinska *et al.* [2004]. There were no significant differences in **HMF** concentration in milk stored at 4 and 8°C, but storage at room temperature caused a two fold increase in its amount when compared with freshly sterilized product. **HMF** concentration was strongly correlated with milk colour changes [Cais-Sokolinska *et al.*, 2004].

Other food products

Several traditional Spanish syrups were analysed for **HMF** palm (*miel de palma*), must (*arrope*), sugarcane syrup (*miel de caña*) as well as molasses [Ruiz-Matute *et al.*, 2010]. The highest concentration of **HMF** was found in must syrup (3500–11000 mg/kg). Molasses and sugarcane syrups were characterized by much lower amounts of **HMF** (100 and 100–300 mg/kg, respectively). The **HMF** concentration in palm syrup was below 3 mg/kg [Ruiz-Matute *et al.*, 2010].

Husoy *et al.* [2008] investigated the **HMF** concentration in 35 different samples of Norwegian food, and a level of **HMFA**, an **HMF** metabolite, in human urine. **HMFA** level in urine was correlated with a daily **HMF** intake from food. The highest **HMF** amount was detected in: coffee (91.3–3060 mg/kg), prunes (237 mg/kg), dark bear (13.3 mg/kg), canned peaches (5.8 mg/kg) and raisins (5.0 mg/kg). Surprisingly, a lower concentration of **HMF** was found in bakery products and in breakfast cereals (0.06 up to 0.65 mg/kg) [Husoy *et al.*, 2008]. This appears to be in opposition with the results discussed earlier [Ramírez-Jiménez *et al.*, 2000a, b; Rufían-Henares *et al.*, 2006; Ameur *et al.*, 2007; Gökmen *et al.*, 2007, 2008; Capuano *et al.*, 2009]. These differences may have resulted from both the specificity of the tested products (technology and/or composition) as well as the different methodologies used. In the case of bakery products, the researcher should clearly identify what portion of baked goods is tested for **HMF**. Important here is for example the crust to crumb ratio.

The kinetics of **HMF** formation in Chinese rice wine was studied by Chen *et al.* [2010]. The authors carried out analysis of raw rice wine, and wine after ethanol, phenols, fats and protein extraction. **HMF** formation in raw samples followed first-order kinetics contrary to samples after extraction, which were described by zero-order kinetics. The activation energies were 43.0, 123.9, and 89.1 kJ/mol, respectively [Chen *et al.*, 2010].

Theobald *et al.* [1998] investigated the possibility of **HMF** formation during vinegar production. 220 vinegar samples including those of malt, sherry, white and red wine, apple balsamic as well as table vinegar with caramel were analysed. The **HMF** level was found to be very low except in balsamic vinegar and ranged from 316 to 3250 mg/kg. Vinegar is usually stored in wooden barrels for up to 25 years. According to the authors, **HMF** concentration seems to be a good indicator of the storage age of balsamic vinegar [Theobald *et al.*, 1998].

HMF METABOLISM AND ITS IMPACT ON ORGANISMS

Although **HMF** is a well-known by-product of thermal processing, its impact on human health is still a contentious topic. There is much debate over its toxicity, genotoxicity, mutagenicity, and carcinogenicity. Reports showing a protective role for **HMF** are also available, putting its supposed toxicity into question.

Daily intake

Humans are potentially exposed to **HMF** through pharmaceutical preparations, cigarette smoke, and the consumption of some beverages and foods. Data regarding the daily average intake of **HMF** is very limited. Rufían-Henares & de la Cueva [2008] estimated the daily dietary intake of **HMF** in the Spanish population. The potential **HMF** exposure was calculated for three different scenarios by using individual food intake and the minimum (scenario 1), median (scenario 2) and maximum (scenario 3) values of analytical data on the **HMF** content in food. A mean **HMF** intake of 10 mg/day (corresponding to scenario 2) was obtained, with coffee and bread being the most important contributing food items (85% of the total **HMF** daily exposure) [Rufían-Henares & de la Cueva, 2008].

In 2008, a study of **HMF** dietary intake was performed for the Norwegian population by means of 24-h recall and comparison with the level of **HMFA** in urine [Husoy *et al.*, 2008]. The 95th percentile of the estimated daily dietary intake of **HMF** and the 24-h urinary excretion of **HMFA** were 27.6 and 28.6 mg, respectively. Although there was a significant correlation ($r=0.57$, $P<0.001$) between the estimated **HMF** intake and urinary **HMFA**, most participants of the study had lower estimated **HMF** intake than the amount of **HMFA** excreted in urine, which suggested some other sources of **HMF** exposure.

Both above-mentioned reports showed that the daily intake of **HMF** was significantly lower than estimated by Ulbricht *et al.*, [1984] which equalled 150 mg/person. The spectrophotometric methods of **HMF** determination used by Ulbricht however, could overestimate the levels of **HMF**.

As alternative sources of **HMF**, cigarette smoke and some pharmaceutical preparations should be considered. The pyrolytic breakdown of cellulose in cigarettes is believed to generate **HMF** amongst other furans [Wieslander *et al.*, 1993]. **HMF**, as a product of glucose and fructose thermal degradation, is also present in many medical solutions used for parenteral nutrition, peritoneal dialysis (PD), and intravenous injections which are heat-sterilised [Nilsson-Thorell *et al.*, 1993].

The **HMF** level in a 50% dextrose injection, within 24 h of manufacturing, was 720 mg/kg, but after four years of storage at 21°C it had reached 5800 mg/kg [Murty *et al.*, 1977]. In sterile glucose solutions, **HMF** concentrations of about 1 to 90 mg/kg have been reported [Ulbricht *et al.*, 1984]. Inverted sugar or glucose-containing parenteral solutions have been reported to have **HMF** concentrations ranging from 3 to 56 and 1 to 4 mg/kg, respectively. **HMF** concentration correlated positively with high acidity ($pH<4$), higher sterilisation temperature (>110°C) and a longer sterilisation time (30 min). **HMF** and other decomposition products of fructose had been detected at considerable amounts (up to 1200 mg/kg) in fructose-containing solutions for intravenous injection [Jellum *et al.*, 1973; Wieslander *et al.*, 1993].

From the clinical standpoint, the concentration of **HMF** in parenteral solutions does not seem to pose any significant toxicological risk. **HMF** is rapidly metabolised and excreted with urine. A patient on peritoneal dialysis (PD) uses between 8 and 20 L of dialysis fluid every day depending on the treatment regime resulting in the consumption of 3–7 tons of fluid with 1.5–4.0% glucose (50–175 kg pure glucose) *per annum*. Taking into consideration the notably high local exposure of the cells within the peritoneal cavity to these fluids, the presence of contaminating substances such as **HMF** and their impact on human cells should be of considerable interest.

Metabolism of HMF

There are only a few reports on the absorption, transport and metabolic pathways of **HMF** in humans. Delgado-Andrade *et al.* [2008] examined **HMF** availability using an *in vitro* model of the human intestine. They evaluated the transport of **HMF** at different concentrations across the Caco-2 cells monolayer and observed that the absolute value of trans-

ported **HMF** was positively correlated with **HMF** concentration in media. This direct relation was not maintained when results were expressed taking into account the initial amounts placed in the apical chambers. The authors suggested also that food composition influences the **HMF** uptake in the intestine [Delgado-Andrade *et al.*, 2008].

Up to date, there is no consensus regarding the metabolic pathway of **HMF**. It is possible that the degradation of **HMF** and its metabolites in bacteria employs different routes than those observed in higher organisms. Moreover, some **HMF** metabolites were found in humans but not in rodents. The possible pathways of **HMF** biotransformation postulated by different authors are presented in Figure 6 and described below.

Koopman *et al*. [2010] identified **HMF** and furfural metabolic pathways in Gram-negative bacteria *Cupriavidus basilensis* **HMF**14, and isolated and characterised the genes involved in those reactions. The unique enzyme essential for furfural degradation, encoded by *hmfE*, is likely a 2-oxoglutaroyl-CoA-thioester hydrolase. The authors demonstrated that degradation of **HMF** in bacteria proceeds *via* 2,5-furan dicarboxylic acid (**FDCA**), and requires an FAD-dependent oxidoreductase, encoded by *hmfH*. Hence, the enzyme HmfH oxidises **HMF** to 5-hydroxymethyl-2-furoic acid (**HMFA**), and then to **FDCA**. It was shown that the enzyme could also oxidise **HMF**, **HMF alcohol**, 2-furanmethanol (**FM**) and furfural (**F**) to their corresponding monocarboxylic acid forms. **FDCA** is decarboxylated to 2-furoic acid (**FA**), which is then metabolised by the furfural degradation route [Koopman *et al*., 2010]. Akıllıoglu *et al*. [2011] observed that **HMF** was reduced during wort fermentation by yeasts. In this process, **HMF** was converted into **HMF alcohol**, and its degradation was more rapid than either glucose or fructose. **HMF** degradation occurred faster when there was sugar in the fermentation medium.

Studies with radiolabelled [^{14}C]-**HMF** showed that 5-hydroxymethyl-2-furfural was rapidly absorbed in the gastrointestinal tract in male B6C3F1 mice and F344 rats [Godfrey *et al.*, 1999], and that tissue concentrations in male mice at the earliest observed time point were not linearly proportional to dosage. Excretion of **HMF** was primarily *via* urine with an efficiency of 60–80% of administered **HMF** excreted by this route within 48 h. The increased level of **HMF**-derived radioactivity was observed in the liver and kidney. Similar results were obtained by Germond *et al.* [1987] in their study performed also with radiolabelled [^{14}C]-**HMF** in rats. The authors demonstrated that **HMF** or its metabolites were rapidly eliminated in the urine with a recovery of 95–100% after 24 h.

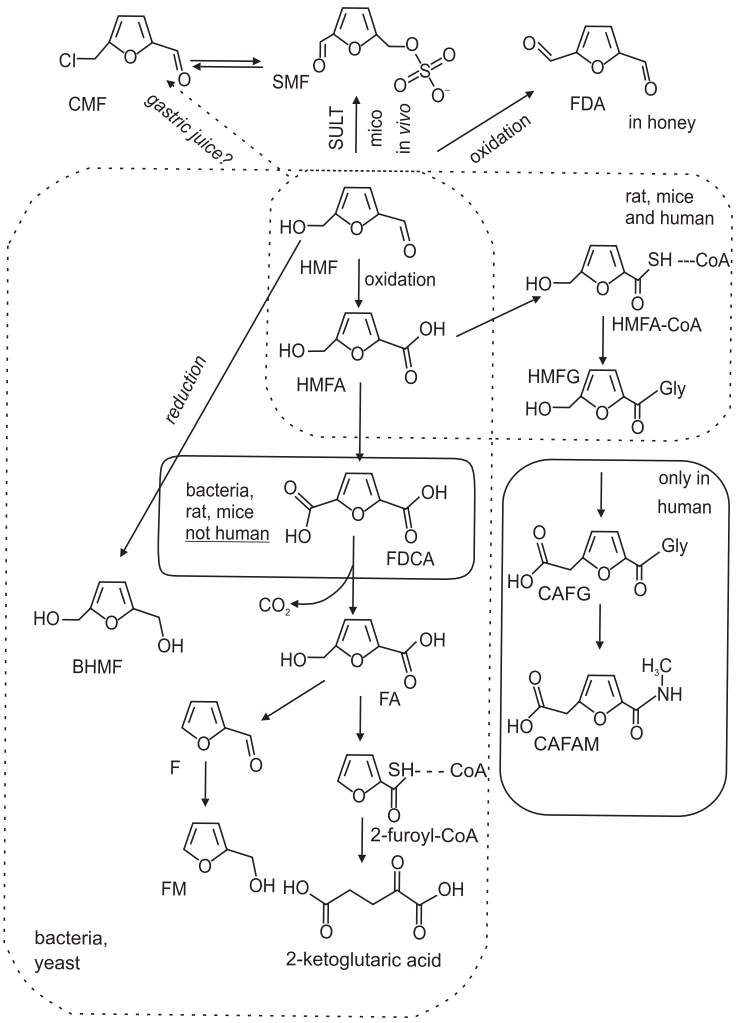

FIGURE 6. Pathways of **HMF** biotransformation.

They postulated that **HMF** was excreted in rat urine mainly as **HMFA** and its glycine conjugate N-(5-hydroxymethyl-2-furoyl)-glycine (**HMFG**). Both are the products of oxidation pathway of **HMF**, and the formation of **HMFG** was inversely proportional to **HMF** dose in rats but not in mice. Whole-animal-body autoradiography confirmed that shortly after administration, the radiolabelled material was present in the liver but mostly in the kidney and the bladder [Germond *et al.*, 1987; Godfrey *et al.*, 1999].

These results are not in accordance with observations made earlier in humans suggesting that different biotransformation pathways may occur in humans and rodents. Jellum *et al.* [1973] analysed urine samples obtained from two infants that underwent surgical operations, before, during and after receiving parenteral nutrition solutions, which contained **HMF**. They calculated that 38% (in one case) and 74% (the second case) of administered **HMF** was excreted *via* the urine as the **HMF**-derivatives: **HMFA** and 2,5-dicarboxylic acid (**FDCA**). There were neither **HMF** nor the glycine conjugates of **HMFA** and **FDCA** present in urine. The authors suggested that the remaining **HMF** was probably retained in the body and was bound to proteins. However, Jellum *et al.* [1973] could not find **HMFG** (the glycine conjugate of **HMFA**) in urine because they performed ether extracts on acidified urine and **HMFG** is not soluble in ether. Interestingly, that increasing **HMF** dose results in increasing of an **HMFA**/**HMFG** ratio [Germond *et al.*, 1987]. This may suggest that **HMFG** formation is impeded by the availability of free glycine. The lack of glycine results in the excretion of free 2-furoic acid (**FA**) or **FDCA** generated through the second pathway.

In the experiments of Prior *et al.* [2006], four metabolites were identified in human urine and plasma after consumption of dried plum juice, being rich in **HMF**. The major metabolite, identified as the oxidation product of **HMF**, was **HMFA**. The amount of this compound excreted in the urine in the first 6 h after consumption of dried plum juice was 1465 μmol (36.9% of the dose of **HMF**). **HMFA** was also a significant metabolite of **HMF** present in human plasma with the maximum level observed after 30 min. Other metabolites identified in urine, based on HPLC-MS/MS results, were: 5-hydroxymethyl-2-furoylglycine (**HMFG**), (5-carboxylic acid-2-furoyl) glycine (**CAFG**), and 5-hydroxymethyl-2-furoyl aminomethane (**CAFAM**), with recovery levels of 3.4%, 4.2%, and 1.8% respectively. **CAFG** and **CAFAM** were also present at detectable levels in urine of the control subjects. No evidence was obtained to illustrate the formation of **FDCA** in humans.

It was postulated that **HMF** that had escaped digestion in the gut could have been transformed to 2,5-bis(hydroxymethyl)-furan (**BHMF**) by intestinal microflora [Boopathy *et al.*, 1993].

Carcinogenic, toxic and mutagenic activities of HMF

It is not clear whether **HMF** represents a potential health risk. **HMF** is considered an irritant to the eyes, upper respiratory tract, skin and mucous membranes. Data from case reports and epidemiological studies showing correlation between exposure to **HMF** and risk of cancer development in humans is not available. Studies on rat and mice, however, have indicated potential carcinogenic properties of **HMF**.

HMF can initiate and promote the growth of aberrant crypt foci (ACF) in rat colons in a dose-dependent manner [Zhang *et al.*, 1993]. Furfural and 5-hydroxymethyl-2-furfural induced a significant number of chromosome aberrations and a significant lowering of mitotic activity in cultured Chinese hamster V79 cells [Nishi *et al.*, 1989]. In a two year study conducted by the National Toxicology Program, **HMF** was found to increase the incidence of hepatocellular adenomas in female B6C3F1 mice, whereas no carcinogenic activity was observed in male or female F344/N rats as well as in male B6C3F1 mice [NTP Technical Report, 2010]. In addition, **HMF** was associated with increased lesions of the olfactory and respiratory epithelium of the nose in male and female rats and mice [NTP Technical Report, 2010].

Conversely, Rasmussen *et al.* [1982] could not demonstrate any adverse effect of **HMF** (400 mg, injected subcutaneously, twice per day for one week) on the following parameters: weight, haemoglobin, leucocytes, platelets, serum-protein, serum-alanine-aminotransferase, alkaline phosphatase, liver cell necrosis and hepatic steatosis as compared with the control group. Moreover, the addition of a 200 mg **HMF**/L isotonic NaCl solution did not increase the vein irritating effect of the solution, when given as a 5-h continuous intravenous infusion. The amounts of **HMF** used in this study far exceeded doses typically received by patients from glucose solutions.

Shinohara *et al.* [1990] evaluated the effects of **HMF** on the viability and activity of some enzymes in U-937 cells (human histiocytic lymphoma cell line). The cells were incubated in different concentrations of **HMF** (3.9–117 nmol/L) for 12 h. Incubation of U-937 with the highest dose of **HMF** resulted in a 20% reduction in cell viability whilst lower concentrations of **HMF** had no effect. Authors reported a dose-related increase in the activity of NADPH-cytochrome c reductase at all concentrations of **HMF**, and no effect of **HMF** on the activity of glutamic oxaloacetic transaminase.

When human blood cells were incubated in a different concentration of **HMF** the heat output was increased by approximately 60% in erythrocytes at an **HMF** concentration of 7.35 mmol/L. An adverse effect was seen in granulocytes where a statistically significant reduction in heat output, of about 17%, was found. No influence of thrombocytes on the metabolic activity could be detected [Nässberger, 1990].

It was postulated that **HMF** could be metabolically-activated *via* esterification of the hydroxyl group [Surh & Tannenbaum, 1994]. The chemically-synthesised sulphuric acid ester, 5-sulfooxymethyl-2-furaldehyde (**SMF**), exhibited direct mutagenicity in human lymphoblasts and induced 8-azaguanine-resistant mutants in *Salmonella typhimurium TM677* in a dose-dependent manner. **SMF** also induced dose-dependent increases in the number of His+ revertants in *Salmonella typhimurium* TA100 [Surh *et al.*, 1994]. **SMF** may cross-link target cell DNA thereby exerting its toxic effects. The 5-sulfooxymethyl group can covalently bind to DNA bases by the S_N1 or S_N2 mechanism, and the C_2 aldehyde functional group may interact with other nucleophilic sites on DNA [Lee *et al.*, 1995]. Moreover, the mutagenicity

of **SMF** could be enhanced by the addition of extra chloride ions to the assay medium. The product of this allylic chlorination, 5-chloromethylfurfural (**CMF**), was more mutagenic and cytotoxic in bacteria than **SMF** [Surh & Tannenbaum, 1994]. When **SMF** and **CMF** were topically applied to mouse skin, higher skin tumour-initiating activity was observed than with the application of **HMF**. 5-Chloromethylfurfural was found to be a strong hepatocarcinogen in infant male B6C3F1 mice [Surh *et al.*, 1994].

It was demonstrated that **SMF** exerted a strong nephrotoxic effect in male FVB/N mice *in vivo*. Mice which received single doses of **SMF** (250 mg **SMF**/kg body mass, i.p.) died or were moribund 5–11 days after the treatment. Histopathological analyses revealed that **SMF** induced moderate damage to liver tissue and notable damage to the kidneys (nearly all proximal tubules in **SMF** exposed animals were destroyed). The molecular mechanism underlying this selective toxicity of **SMF** for proximal tubules is unknown [Bakhiya *et al.*, 2009].

Recently, *in vivo* studies by Monien *et al.* [2009] first proved that **SMF** is formed in **HMF**–treated mice. The maximum **SMF** plasma level was observed at the first sampling time, 2.5 min after **HMF** administration. On the basis of these kinetic data, it was estimated that between 452 and 551 mg/kg of the initial **HMF** dose (500 mg/kg) was converted into **SMF** which was subsequently circulated. The Authors suggest also, that **SMF** plasma concentrations in mice probably underestimate the actual level of **HMF** sulfoconjugation because **SMF** may react partially with proteins and DNA close to the site where it had formed.

5-Hydroxymethyl-2-furfural has been tested for mutagenicity in different bacterial and mammalian test systems, and the results indicate low or no mutagenic effect of **HMF**. Severin *et al.* [2010] demonstrated that **HMF** was not cytotoxic to bacteria at the highest concentration (5000 μg/plate), with or without exogenous activation system (S9). In the study, none of the results of the Ames test (+S9 or −S9) exceeded the critical value of 2.0 and all quotients ranged below 1.6, except at the lowest concentrations (0.5, 5 and 50 mg) in TA 1535 with S9 (2, 1.7, 1.6, respectively for the quotients), without concentration effect profile. Therefore, the Ames test did not show any genotoxic potential of **HMF** compared to the respective positive controls [Severin *et al.*, 2010]. In studies conducted by the NTP, 5-hydroxymethyl-2-furfural was weakly mutagenic to *S. typhimurium* strain TA100 in the absence of exogenous metabolic activation (S9) over a concentration range of 100 to 10,000 μg/plate. No mutagenic activity was detected in TA100 with S9 or in strains TA97, TA98, TA102, or TA1535, with or without S9 [NTP Technical Report, 2010].

It is possible that the negative results for **HMF** genotoxicity in standard activating systems are observed because they disregard the enzymes involved in **HMF** conversion to **SMF** – sulfotransferases (SULT). As mentioned above, both **SMF** and **CMF** exerted a mutagenic effect on bacteria.

Experiments conducted on different cells revealed that the role of sulfotransferases in the impact of **HMF** on living cells can be deduced. Severin *et al.* [2010] estimated the viability of HepG2 cells exposed to different concentrations of **HMF** using the Alamar Blue assay. The authors demonstrated the cytotoxicity of **HMF**, with an IC_{50}=38 mmol/L, suggesting weak toxicity. In the same study, the genotoxic effect of **HMF** was evaluated using the comet assay. The OTM for **HMF**-treated HepG2 cells increased significantly with a concentration-effect profile from 7.87 to 36.6 mmol/L. This indicated that **HMF** induced DNA breakage in the tested cells.

This data is not in accordance with the studies of other authors. Janzowski *et al.* [2000] did not observe any DNA damage after 1-h exposure to **HMF**, up to the cytotoxic concentration limit (80 mmol/L, 75% absolute viability) using the comet assay on V79 or Caco-2 cells. The discrepancies between those studies may be due to the lack of the sulfotransferase in Caco-2 or V79 cells and its presence in HepG2. Cytotoxicity (trypan blue exclusion) of **HMF** was also investigated. The authors designated LC_{50}. The results of trypan blue exclusion indicated that **HMF** had a moderate influence on V79 (LC_{50}=115 mmol/L) and Caco-2 (LC_{50}=118 mmol/L), they also demonstrated the cytotoxicity, with IC_{50}=6.4 mmol/L, which suggests weak cytotoxic activity. Janzowski *et al.* [2000] studied the mutagenicity of **HMF** in V79 HPRT assay *in vitro* as well. V79 Chinese hamster cells have one functional copy of the gene, which codes for the HPRT enzyme (hypoxanthine-guanine phosphoribosyltransferase). The HPRT assay results showed that **HMF** was weakly mutagenic at the *hprt*-locus in V79 cells, however **HMF** did not cause abnormal growth of cells. The results of Janzowski *et al.* [2000] suggest that **HMF** does not pose a serious health risk to human health.

Total glutathione was determined in V79 cells, Caco-2 by photometric determination of 5-thio-2-nitrobenzoate (**TNB**), formed from 5,5'-dithiobis(2-nitrobenzoate acid) (**DTNB**). **HMF** induced a concentration-dependent glutathione decrease in both cells. Therefore, these results suggest that **HMF** could have a negative influence on human health [Janzowski *et al.*, 2000].

Durling *et al.* [2009] also performed the comet assay to evaluate the DNA-damaging effects of **HMF** in several cell lines characterised by different activities of the enzyme sulfotransferase SULT1A1. The authors used two human cell lines (Caco-2, low activity; and HEK293 with higher activity of SULT1A1), one cell line from mouse (L5178Y, no activity) and two cell lines from Chinese hamster (V79, negligible activity; and V79-hP-PST, high activity of human SULT1A1). The genotoxic effect of **HMF** could be observed in those cells only at high concentrations (100 mmol/L, 3 h of exposure) and it was usually associated with concomitant decreased cell viability after using the trypan blue exclusion test. The damaging effect of **HMF** at a lower concentration (25 mmol/L) was observed only in cell lines expressing high sulfotransferase SULT1A1 activity [Durling *et al.*, 2009].

Human SULT isoforms have a widespread tissue distribution and are expressed in many tissues including liver, lung, brain, skin, platelets, breast, kidney, and gastrointestinal tract [Salman *et al.*, 2009]. Moreover, humans express SULT in extrahepatic tissues more extensively than rodents do and may therefore be more sensitive to **HMF** [Teubner *et al.*, 2007]. This suggests that the risk associated with a high intake

of **HMF** from food may be higher for humans than indicated by experiments using rodents.

Some studies on mutagenicity or carcinogenicity of other **HMF** derivatives are available as well. Furfuryl alcohol and furfural were not observed to be mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, or TA1537, with or without S9 [Aeschbacher *et al.*, 1981; Shinohara *et al.*, 1986; NTP Technical Report, 1990, 1999]. 2-Furoic acid also gave a negative result when tested in an Ames/*Salmonella typhimurium* assay using strains TA98 and TA100 [Ichikawa *et al.*, 1986].

The lack of mutagenic as well as genotoxic activities of **HMF** was also stated on the basis of *in vitro* studies on bacterial systems with and without an activation factor S9 [Florin *et al.*, 1980; Kasai *et al.*, 1982; Kim & Richardson, 1992].

Hiramoto *et al.* [1996] investigated strains of *S. typhimurium* TA 100 and TA 98, treated with an activation factor. Authors did not observe any mutagenic activity associated with **HMF** but certain cytotoxicity was found [Hiramoto *et al.*, 1996].

Furfuryl alcohol did not induce sister-chromatid exchanges (SCEs) in human lymphocytes [Jansson *et al.*, 1986] and in cultured Chinese hamster ovary cells in the presence of S9, but it did so in the absence of S9 [NTP Technical Report, 1999]. No induction of chromosomal aberrations was noted in cultured Chinese hamster ovary cells treated with furfuryl alcohol in the absence of S9 but in the presence of S9 an equivocal result was obtained. Under the conditions of the 2-year inhalation studies, there was some evidence of carcinogenic activity of furfuryl alcohol in male F344/N rats (increased incidences of combined neoplasms of the nose) and in male B6C3F1 mice (increased incidences of renal tubule neoplasms) [NTP Technical Report, 1999]. Furfural was proved to be carcinogenic for male B6C3F1 mice (higher incidences of hepatocellular adenomas and hepatocellular carcinomas) and for female B6C3F1 mice (increased incidence of hepatocellular adenomas) [NTP Technical Report, 1990].

Hadi *et al.* [1989] reported that furfural induced single strand breaks in double stranded DNA (occurring preferentially in AT base pairs), and the evidence showed that the strand scissions induced by furfural in DNA account for its biological activity as assayed by inactivation of bacteriophage lambda.

It should be noted that studies which demonstrate the positive and protective role of **HMF** are also available. Wang *et al.* [2010] revealed that **HMF**, present in traditional Chinese medicine, improved the morphology of H_2O_2-treated human LO2 hepatocytes and inhibited the level of caspase-9 and caspase-3 in the cells. Hence, **HMF** exerted protective activity by preventing oxidative injury and apoptosis in liver tissue. These results were confirmed by Ding *et al.* [2010] who conducted a measurement of cell viability (MTT) on hepatocyte cell line LO2. The cells were exposed to **HMF**, derived from processed *Fructus corni*. This method depends on dye (MTT) reduction, through mitochondrial dehydrogenase to insoluble formazan crystals. The human cells were divided into five groups: control group, H_2O_2 group, and a group containing three concentrations of **HMF** (0.2, 0.5 and 1 μg/mL). According to the obtained results, the authors claimed that H_2O_2 markedly decreased the viability of hepatocyte cell line LO2 (by 40%), while cells exposed to different concentrations of **HMF** were characterised by the better viability. The results suggest that **HMF** protects LO2 cell from oxidative damage. The authors also investigated hepatocyte cell apoptosis and cell cycle by flow cytometric analysis. LO2 cells were exposed to H_2O_2 and different concentrations of **HMF**. Cells were analysed by using a FACSCalibur flow cytometer. The results showed that major apoptosis and DNA degradation were observed in cells exposed to H_2O_2. DNA degradation and apoptotic rate significantly decreased after treatment with **HMF**. This suggests that **HMF** inhibits apoptosis and DNA degradation. The effects of **HMF** on NO release were also observed, with results showing that **HMF** reduced NO release thus inhibiting apoptosis [Ding *et al.*, 2010].

The US patent no 2005/0124684 is based on the discovery that **HMF** inhibited the expression of TNF-α and IL-1β; cytokines involved in many disorders. The suggested methods of treatment of those diseases are proposed in the document by Du *et al.* [2005]. In turn, Uckun *et al.* [2001] identified the usefulness of one of the derivatives of **HMF**, *i.e.* 5-hydroxymethyl-2-furoic acid (**HMFA**) in neoplastic treatment. **HMFA** was found to hamper tubuline polymerization and microtubule formation, thus contributing to mitosis inhibition in cancer cells [Uckun *et al.*, 2001].

When the inhibitory effect of methanolic extracts of *Dictyophora indusiata* against mushroom tyrosinase was proved, the chromatographic and spectroscopic methods were used for identifying the adequate bioactive component. It was shown that **HMF** present in the extract was responsible for the inhibitory effect, and the kinetic studies revealed it to be a non-competitive inhibitor for the oxidation of L-DOPA [Sharma *et al.*, 2004]. **HMF** was also identified as the active component in the hot water extracts of *Lycium Chinese*. It showed an inhibitory effect on β-hexosaminidase release by IgE-sensitised BSA-stimulated rat basophilic leukaemia (RBL-2H3) cells. Moreover, **HMF** suppressed $[Ca^{2+}]i$ influx in the RBL-2H3 cells [Yamada *et al.*, 2011]. It has been suggested that **HMF** could be useful for the treatment or prevention of type I allergic diseases. Another studies demonstrated that **HMF** could be developed as a novel marine natural antioxidant or potential precursor for practical applications in the food, cosmetic, and pharmaceutical fields [Li *et al.*, 2009].

It was demonstrated that **HMF** specifically binds to the N-terminal amino acid of intracellular sickle haemoglobin (HbS) by forming a high affinity Schiff-base adduct with HbS, and thus inhibits red cell sickling by allosterically shifting oxygen equilibrium curves toward the left [Abdulmalik *et al.*, 2005]. **HMF** seems to be a very promising molecule for the treatment of sickle cell anaemia, and has successfully passed phase I, phase IIA and phase IIB clinical trials, in Nigeria. According to the Authors of US Patent no. 7119208 [Safo *et al.*, 2006], 5-membered heterocyclic compounds, among them **HMF**, have a dual mode of action. First, binding of the compounds to haemoglobin increased the oxygen affinity of both normal and sickle haemoglobin. Secondly, binding of these compounds to the N-terminal amino acid of sickle haemoglobin resulted in a destabilization of potential contacts between sickle haemoglobin molecules, pre-

venting polymerisation and the formation of fibrous precipitates of the sickle haemoglobin. **HMF** also induces hypoxia, and thus might be useful to augment cancer treatment.

CONCLUSIONS

Due to the nature of the food industry, especially the chemical composition of processed raw materials, unit operations and processing conditions; the synthesis of **HMF** in foods is very common. It is favoured by the accumulation of not only simple sugars but also polysaccharides, proteins and amino acids, low pH and high temperatures which are required in processing. It seems that strong consideration must be given to the impact of **HMF** on the human health, and as a consequence of this steps should be taken to reduce its level in food. Although recommendations for storage and/or processing of food products have been made, so far no direct efforts have been developed for to reduce the formation of **HMF** in food. Recommendations for the storage of food products or processing temperatures however, may help to lower the potential levels of **HMF** in foods. Changes in the production of certain foodstuffs may entail major changes in the sensory and quality characteristics of the final products. Consequently, the ratio of benefits and losses that may arise as a result of alteration in technological processes should be taken into consideration. Our review indicates that due to the fact that there is inconclusive evidence regarding **HMF's** potential toxicity to human health, it cannot be determined whether **HMF** should be considered unsafe or whether the benefits of its use in industry outweighs the risks it may pose. Additional studies are needed to elucidate the potential effects that long term exposure to **HMF** could have on human health.

ACKNOWLEDGMENTS

Authors would like to thank Mr Alexander Zwolinski for kind help in editing of the paper.

The work was financed by scientific grant No DS 3700/WTZ/2013.

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Submitted: 13 July 2012. Revised: 10 September 2012. Accepted: 17 September 2012. Published on-line: 22 August 2013.