MAILLARD REACTION PRODUCTS IN FOOD AND THEIR POTENTIAL HEALTH IMPACT: A NEW CHALLENGE FOR BEER

Rosa Santella
Birthe Møller Jespersen
Mogens Larsen Andersen

Department of Food Science
University of Copenhagen

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Abstract
During high thermal stress in food, reducing sugars and amino-groups of peptides react, generating non-enzymatic brownish compounds together with peculiar flavor and color. Sources of those Maillard reaction products are extensively consumed in our everyday life: coffee, diary and bakery products, tomato sauce, nuts, chocolate, and beer (especially the darker types). Questions about their influence on health arised as consequences of socio-economic changes, for the increasing amount in the diet of MRPs containing food: less fresh products are ingested, pre-cooked stuff rules in the kitchen, infants have been massively fed with industrial formulas over the last 30 years. Some intervention studies revealed the negative influence on cardiovascular risk of a subgroup known as the advanced glycation end products (AGEs). On the other hand, beneficial biological activities have been associated to the final product called melanoidins such as: hypotensive, anti-inflammatory, anti-oxidants, anti-cancer. However, most of the research has been done in vitro or on animals; then the impact on humans has not been elucidated yet. However, beer MRPs characterization remains still scarce. The old ELISA turned out to be an unspecific method of quantification, instead HPLC and LC-MS more reliable detection tools, although not so many markers are available, therefore a bigger effort is required to target and discriminate them on the matrix, to further investigate their impact on health.

INTRODUCTION

Food and beverage processing meant either as industrial treatment or simple homemade cooking, implies inevitable flavour, aroma and colour release, as consequence of non-enzymatic reactions between reducing sugars and amines in moisture(Hofmann, 1998a). Those sensorial characteristicsare often desired because contribute to taste or palatability and satisfycostumer expectation. The chemist Louis Camille Maillard, in the beginning of XX century, initiated a discussion about the brownish compounds visible in thermal stressed food,thereforethey have been called after him. Their ubiquity in e.g. dairy, bakery, coffee, brewing, wine and chocolate and the abuse of kitchen techniques that favour their formation (like frying or roasting), stimulated the scientific curiosity about the different types, amount in food and bioactivity (Albalá-Hurtado, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1999; Bai et al., 2017; Capuano, Ferrigno, Acampa, Ait-Ameur, & Fogliano, 2008; Coghe, Gheeraert, Michiels, & Delvaux, 2006; Crozier, Ashihara, & TomÃ, 2011; da Silva Ferreira, Reis, Rodrigues, Oliveira, & De Pinho, 2007; Dexter, Matsuo, & Morgan, 1981; Lin et al., 2010; Olano, Calvo, & Corzo, 1989; Sanz, del Castillo, Corzo, & Olano, 2000; Tamanna & Mahmood, 2015; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002). Nowadays, Maillard reaction products (MRPs) are still a sensitive topic in food science, especially for their potential impact on human health. In particular two relevant classes: Melanoidins and AGEs, that in the last 40 years were suspected to be harmful, as they are not present in raw foods (Delgado-Andrade & Fogliano, 2018). Evolutionary, this is arguable considering man's long history of exposure to heated food. As well known, the discovery of fire by our ancestors revolutionized positively their brain development, making food nutritionally more chewable, digestible and bio-available, apart from improving hygienic safety and texture quality (Carmody, Weintraub, & Wrangham, 2011). Anthropologists believe that human digestive apparatus must have evolved to use the Maillard reaction products in an efficient way, otherwise they wouldn´t be innately attracted from their smell, oppositely to the inedible stuff present in nature. Actually, regarding the effect of the consumption of cooked food from a broad perspective, the "pro"seem to win versus the negative aspects (Van Boekel et al., 2010). However, after thousandths of years, many question marks about MRPs and their relation to health, remain still unanswered (R. N. Carmody & R. W. Wrangham, 2009).

MRPs chemical generation proceeds by steps and is time/temperature dependent. The rule of thumb indicates that, Maillard reaction rate doubles every 10° C increase (Ledl & Schleicher, 1990). pH also is an influencing factor; low formation occurs at acidic conditions and it is accelerated by basic value, up to 10 pH (O'Brien, Morrissey, & Ames, 1989). Low water activity promotes MRPs formation. Then short and low temperature cooking method reduce MRPs, together with coldstorage . Interestingly, it seems that MRPs can take days and weeks to be completed, in this way they can be released over long time and use of acidic ingredients like vinegar or lemon juice lower pH. An estimation of melanoidins daily intake has been performed by Fogliano and Morales (2011) who considered coffee and bread as the two essential sources to be taken into account. They established an intake of 1.5gr per day of melanoidins coming from coffee and 6gr per day from bread for average consumers. Along with coffee and bakery products, vinegar, pilsner and black beer, fried meatballs, sweetwine and chocolate are dietary sources of melanoidins that provide around 4gr per day (Obretenov, Ivanova, Kuntcheva, & Somov, 1993).

The aim of this review, isto offer a general picture of the huge relevance connected to Maillard reaction products in Food Science and their reflex on Public Health.From literature emerges that scientists have put a greater effort into coffee and dairy stuff analysis because worldwide consumed by both genders, young and old subjects, although MRPs derived from beer would appear much more suitable topic, since more conspicuous and assorted in brownish molecules. Indeed, the manuscript wants to enhance the poorness and limits of the research about a special thermal stressed beverage like beer, totally based on grain baking. For this reason, the challenge is placed into the idea to exploit the several products coming from different heating treatment on malt, to characterize them and clarify the influence on human health, as it has not been done yet. To accomplish this purpose, a final discussion is dedicated to beer and the theoretical relevance of its MRPs, taking into account the complexity of the matrix due to an either competitive or reinforcing associated role of other bio-active compounds. However, first of all, it is necessary to provide a bigger pictures of MRPs that are a complex and variegated pool of compounds. Hereby, it´s provided a synthetic synopsis on MRPs that covers: Description of origin, formation and classification in category; Analytical methodology(past and currently used); Output of studies carried out so far; Possibility of future

research development on a perfect MRPs containing model as beer. The last section, in fact, represents a personal interpretation of the immense value included into the un-explored beer, a source rich of all the MRPs.

Maillard reaction products (MRPs): An overview on mechanism of formation for a variegated pool of compounds

Hodge[1953] summarized Maillard reaction in three major stages: First, sugars and amino acid condense into color less Schiff bases, afterwards they are rearranged into Amadori 1-amino-1deoxy-2ketose form, in a third extended phase some yellow shades start to appear(Tamanna & Mahmood, 2015). Amino groups are degraded, carbonyls instead undergo strong modification: enolization, condensation, dehydration, cyclization, fragmentation and oxidation(-Bonnefont-Rousselot, 2002; Hayashi & Namiki, 1986;Ikeda et al., 1996). As side effects, the intermediates tend to react with other amino acids creating alfa-amino carbonyl, therefore aromatic heterocyclic compounds like pyrazines and pyrroles are produced; hydroxymethylfurfural (HMF) is one of the main marker. On the other hand, during this step,colour tends to darken and Post-Amadoricategory called advanced end glycation products (AGEs) is dominant; as result of high crosslinking between protein-bound lysine or arginine and alfa-dicarbonyls. At the latest stage, brown heterocyclic nitrogenous molecules-melanoidins-originate; according to the size they are classified as high molecular or low molecular weight(Yeh, Hsia, Lee, & Wu, 2017).The crucial points of Maillard reactions chainare showed in Figure 1.

AGEs have been connected to the aetiology of metabolic syndrome and diabetes. They seem to trigger pro- inflammatory interruptor called RAGEs associated to insulin resistance, blood pressure increase and hyper-glycaemia (Brownlee, 2001; Marchetti, 2009). Another important element belonging to MRPs group, whose health implications goes beyond the scope of this manuscript, has been pointed out as genotoxic and mutagen and deserves a mention. Indeed, reducing sugars and asparagine undergoes Strecker degradation leading to the formation of acrylamide (Mottram, Wedzicha, & Dodson, 2002). The Swedish National Food Administration, in 2002, announced that acrylamide was present in a high concentration in: Potato chips, French fries potatoes, cereal based products(Rice, 2005; Svensson et al., 2003).Despite the fact that the

effect on human health of relatively low level of acrylamide found in foods has not been clearly elucidated, the study of the deleterious effects caused by consuming a diet containing MRPs and other neo-formed compounds has recently re-emerged, then it is necessary to shed some more light on their structure, behaviour and healthy/harmful effect in order to eventually distinguish their roles (Fedric J. Tessier et al., 2010). On the other hand, several biological activities have been associated to melanoidins: antimicrobial, anti-inflammatory, anti oxidants, dietary fibers simulator acting on the gut microflora growth and hypotensive (Carvalho, Ømgendal, Andersen, & Guido, 2016; Jiménez-Zamora, Pastoriza, & Rufián-Henares, 2015; Luevano-Contreras & Chapman-Novakofski, 2010; Monente et al., 2015; Rivero et al., 2005).

For all those reasons, MRPs health implications regarding either beneficial or toxicological effect, are catching the attention of both food technologist and clinical researchers.

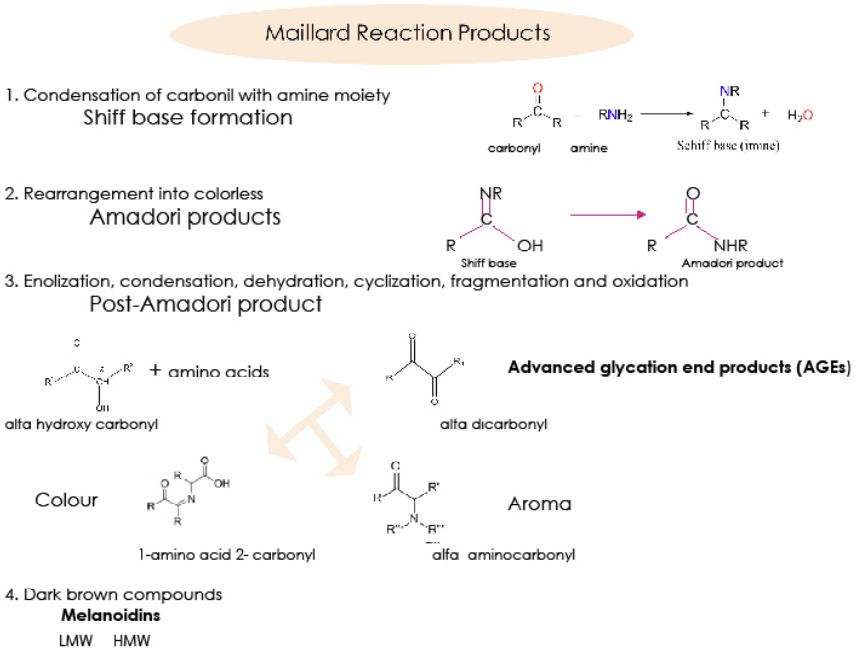

Figure 1.*Schematic Maillard reactions chart.* During an early phase, Shiff bases derived from sugars and amines under heating treatment, are rearranged into Amadori products. In the intermediate stage, from colourless the compounds generated during continuous thermal stress modificationare transformed into yellowish Post-Amadori molecules(AGEs). Typically color and aroma turn to be more evident during this moment also as side effect of secondary products formed; further chemical alterationsoccur until the dark/brown melanoidins appear as final product. The state of art about MRPs health concern exhibits both benefits and potential risks in the Western and Asiatic diet. The first melanoidinss studied where those from soy sauce for their importance into Asiatic diet (Sakurai et al. 1981).Although marginally investigated on their melanoidins content, the sum of all this processed food can be estimated as an average intake of 10 g/ day according to the current occidental dietary habits (Rufián-Henares & Morales, 2007).

Melanoidins: "The forgotten class of food polymers" and their potential as nutraceutics

Melanoidins are called also "the forgotten class of food polymers"-this was the title assigned to the heterogeneous population of compounds, back in 1999, by the European COST Action 919 (Martins & van Boekel, 2003). Contradictory evidences have been shown so far, and in the recent years new insights about the potential health effects raised. It seems that thermal stress responsible for the generation of melanoidins requires temperature of at least 70-90-150 ° Celsius(Ames, 1990). The duration of processing instead generally goes from 35 minutes up to many hours. Melanoidins are quite hydrophilic (Migo et al 1997) and negatively charged (Morales et al., 2002). The rough size estimation range from few thousands of Dalton to 100kDa. The isolation of pure melanoidins fraction is pretty complex(Bailey & Ames, 1998). As melanoidins have different nature in different foods, several approaches have been proposed to estimate their dietary intake(Ames, Royle, & Bradbury, 2000). Over last decade, it has been tried to exploit the physical-chemical proprieties based on solubility, charge, metal chelating activity and hydrophobic. Although the huge research carried out about, melanoidins characterization remains a challenge. Coffee melanoidins were quantified gravimetrically after dialysis,ultrafiltration,and gel filtration (Fogliano&Morales2011),or by spectrophotometer using a specific extinction coefficient(Bekedam, Schols, Van Boekel, & Smit, 2006). A similar approach was used for other water-soluble melanoidins in vinegar, soy sauce, and beverages such as dark beer and sweet wines byTagliazucchi and Bellesia (2015).The literature has always separated them into low and high molecular weight types (respectively LMWs and HMWs). LMWs includes aldehyde, ketones, dicarbonyls, acetamides and heterocyclic compounds. Infact, for example furans and pyrroles present both chromophores and peculiar flavor (Wang, Qian, & Yao, 2011). Different hypothesis of origin have been associated to HMWs that might find an explanation in:

- Polymerisation of LMW as roasting time dependent process(Hayase, Usui, & Watanabe, 2006);
- Crosslink of LMWs and reactive amino acid chains like lysine, arginine, cysteine(Hofmann, 1998b);
- Melanoidins skeleton coming from sugar degradation products are

brunched with amino groups; this is followed by aldol condensation of α-dicarbonyl compounds (Cämmerer, Jalyschko, & Kroh, 2002).

As mentioned above, among all the Maillard reaction products, potential health effects in vitro or on animals have been proposed and most of the research so far focused on coffee (Esposito et al., 2003). Among the most relevant positive consequences emerges :

Anti-glycative activity; Glycation end- products are involved in diabetes complications, thus mechanism preventing their formation are of great interest as potential therapeutics for researchers. Apparently the concentration in the blood of coffee drinker melanoidins could represent an effective anti-glycative dose. It has just been tested on bovine serum albumin and could be diverse according to size and formation step. Post-Amadori products seems overall very efficient (Verzelloni, Tagliazucchi, Del Rio, Calani, & Conte, 2011).

*Anti-inflammatory activity;*Several biomarkers have been taken from the liver of rats after high fat level diet. Rats drinking coffee and melanoidins presented higher antioxidant marker as interleukin-4 and reduced pro-inflammatory cytokines: TNFalpha and interferon gamma. Therefore, it suggestes that melanoidins in coffee may exert anti-inflammatory effects and prevent liver diseases such as non-alcoholic steatohepatitis(Vitaglione et al., 2010).

Anti-hypertensive activity; Melanoidins can influence angiotensin I converting enzyme, controlling ACE inhibitory activity. ACE enzyme catalyses the cleavage of angiotensin I to release a potent vasopressor: angiotensin II; it is a key regulator of renin-angiotensin system that regulates the blood pressure, therefore ACE inhibitors are important for hypertension treatment. This power is enhanced in higher molecular weight melanoidins types and pulled up by high roasting degree. The melanoidins could act chelating the zinc necessary for ACE activity or bind and deform the enzyme behaving as a kind of competitor; even here just in vitro studies have been conducted (Rufián-Henares & Morales, 2007).

Other melanoidins mechanisms of action can be related to anti-microbial power and may consist in the disruption of pathogenic bacteria membrane or interruption of enzymatic reactions vital for their existence (Tranget al.2013). In particular the modulation of the colon microflora place melanoidins at the

level of fibers with prebiotic proprieties, since they increase the number of the desirable Bifidobacteria and Lactobacillus, hampering instead the pathogenic Salmonella and Eschierica coli growth; for this reason they might be protective against colon cancer risk (Jaquet et al. 2009; Picard et al., 2005; Thuoy et al., 2006). In fact, in more recent studies coffee melanoidins seems to increase acetate and propionate in the colon after microbial degradation (López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016). Another mechanism of action played by melanoidins could be the binding of harmful dietary components and elimination as non digestible material (Chandra & Yadav, 2010). Also in the stomach they might fight Helicobacter pylori colonization which causes ulcer and cancer (Hiramoto et al., 2004). In coffee studies again it was found that they could improve dental caries protection through anti-adhesive effect on Sterptococcusmutans that tend to adhere to the tooth surface and tailor biofilm (Daglia et al., 2010). The overall antioxidant capacity could be also due to the interaction with polyphenol structures and the antimicrobial capacity could depend on their zinc and magnesium chelating activity which exhibit radical scavenging power and causes lower redox oxygen species generation (ROS) that are involved in aging and chronic diseases pathogenesis(Morales, Fernández-Fraguas, & Jiménez-Pérez, 2005; Yen, Wang, Chang, & Duh, 2005).

The limiting factor during melanoidins analysis is represented by technical issues in the assessment of the theirstructure and amount in the processed food and the absorbability needed to determine their impact on the Western diet. Even though we are referring to orders of grams and milligrams, it would be still affective in permeating the gastrointestinal tract reaching the bloodstream. Once studies will shed some lights on their different nature, composition, transformation in each food matrix during production and afterwards the transit in the human metabolism, the future challenge may be to create a selective technological system to control the quantitative formation and optimize the thermal treatment favoring beneficial or inhibiting harmful species (Hull, Woodside, Ames, &Cuskelly, 2012).

AGEs: The other side of the medal; harmful bioactive components called glycotoxins

Post-Amadori product in Maillard reactions cascade are labeled under the name

of: Advanced glycation end-products that contain amain modified structures of two basic amino-acids residue: lysine and arginine(S. Reddy, Bichler, Wells-Knecht, Thorpe, & Baynes, 1995). So far, more than 20 kinds ofcompounds have been identified but not all are used for analysis. After Post-Amadori compounds generation, degradation or transformation of di-carbonyls, the starting points are glyoxals and deoxyglucosones.Nε-Carboxymethyllysine (CML) is the first and best characterized AGE in vivo and can be formed through various pathways, for example by condensation of glucose with the ε-amino group of lysine, where the Amadori rearrangement product fructoselysine (Ahemed et al., 1986). This is produced as an unstable intermediate and subsequently undergoes oxidation to form CML. Another pathway depends on the reaction of GO with the e-amino group in lysine (Fu et al., 1996);Nε-Carboxyethyllysine (CEL) is a homologue of CML that is formed by the analogous reaction of lysine residues in proteins with methylglyoxal (Ames et al., 2008a). Methylglyoxal (MG) is one of the most highly reactive carbonyl species (RCS) in the human body (Mathys, Ponnampalam, Padival, & Nagaraj, 2002). Nδ-(5-Hydro-4-imidazolon-2-yl)ornithine (glyoxal derived hydroimidazolone; G-H1) and Nδ-(5-hydro-5-methyl-4imidazolon-2-yl)ornithine (methylglyoxal derived hydroimidazolone; MG-H1) are formed from the reaction of arginine residue with glyoxal or methylglyoxal, and MG-H1 is the major glycation adduct in physiological systems. Argypyrimidine instead is originated from arginine (Heijst et al., 2005)(N. Ahmed, Dobler, Dean, & Thornalley, 2005).

In the AGEs chemical pattern, several cross-linkers have been identified, such as lysine dimers resulting from the reaction between two lysine side-chains and two molecules of glyoxal (GO), methylglyoxal (MG), or 3-deoxyglucosone (3-DG), named GOLD, MOLD, and DOLD, respectively, together with cross-links between arginine and lysine, named GODIC, MODIC, and DODIC (Ahmed et al., 2003). Pentosidine is an imidazo-pyridinium compound derived from lysine and arginine residue cross-linked by ribose and has been used as an indicator of glycation because this compound is stable for acid hydrolysis (Sell et al., 1989). Besides, AGEs are distinguished according to paired fluorescent and crosslinking proprieties as well: for istance, pentosidine is a brown and fluorescent crosslinking substance, glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) are non-fluorescent crosslinking products, whereas both non-crosslinking and non-fluorescent adducts are represented by

carboxymethyllysine (CML) and pyrraline (a pyrrole aldehyde) (S. Rahbar and J. Figarola et al., 2002 ; K. Ikeda et al., 1996). Another frequently measured compound is furosine, which is formed by acid hydrolysis of the protein bound Amadori product of lysine (Krause et al., 2003). Strecker aldehydes are final Maillard reaction products released as the aromatic furfurals. A description of AGEs pathway generation in a simplified model is showed in Figure 2.

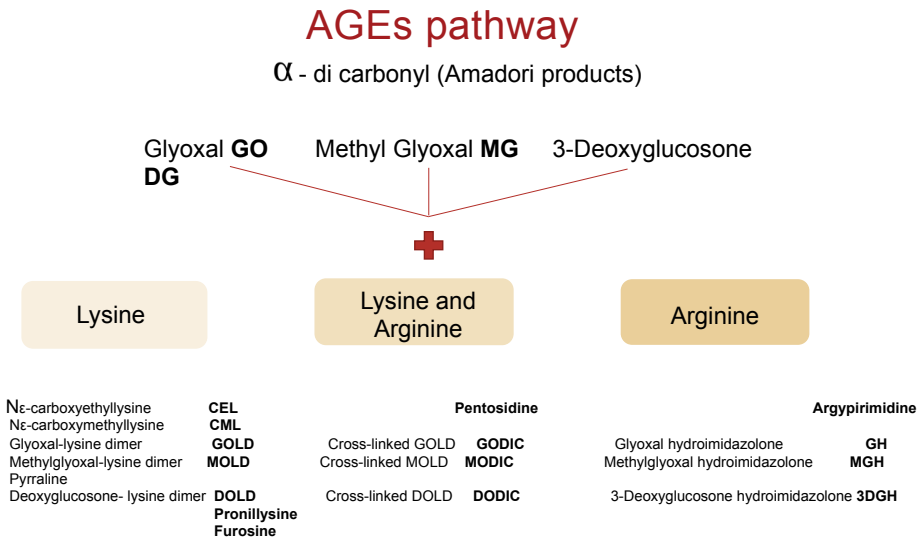

Figure 2. Pathway for AGES formation. In an initial phaseα-di carbonyls Post- Amadori products are chemically transformed into sugar derived degradation products -glyoxals and deoxyglucosones- that are going to react with amino acids arginine and lysine or both generatingsecondary products. In this figure they are sorted out into columns.

Exogenous dietary AGEs has been suggested to increase the in vivo pool of AGEs after intestinal absorption, and contribute to the development of diabetes and related complications (Uribarri et al., 2005). In addition to investigating the effects of AGEs, recent studies have also addressed the effects of MG on many chronic and aging-related diseases in clinical practice (Rabbani, Xue, & Thornalley, 2016). Indeed, MG concentration in the blood of DM patients is reported to be significantly higher than that in non DM patients (Lapolla et al., 2003). Under study is the hypothesis that polyphenols can inhibit the bio-synthesis of AGEs through their antioxidant properties, metal-chelating ability, protein interaction, MG trapping, and/or blocking the receptor for advanced glycation end products (RAGE) (Tan et al, 2008; Xie et al., 2013). Polyphenols with a certain relevance are: phenolic acids, stilbenes, lignans, and flavonoids. Furthermore anti-glycation functions found in recent years were examined to estimate the neutralization or compensation ability against AGEs (Yeh et al.,

2017).

▶ *Proposed toxic effect of AGEs*

AGEs suspected action can cause tissue damage interfering with protein function.They create intra molecular link with the tissue itself, promote free radical formation, and induce inflammatory response after binding receptors called RAGE. There they trigger the pro-inflammatory pathway via mediator recruitment such as cytokines, switching off AGER1 anti-inflammatory receptor(Ramasamy, Yan, & Schmidt, 2011). Moreover, free radicals generated during the glycation process were found to be able to deplete nitric oxide which may result in endothelial dysfunction and vascular thickening(Uhlmann, Rezzoug, Friedrichs, Hoffmann, & Wiedemann, 2002). At the same time, the production of cytokine and growth factor induced by the interaction between AGE-modified proteins and AGE receptors could support the development of atherosclerotic plaques.(Westwood, Argirov, Abordo, & Thornalley, 1997; Yang et al., 1994).

▶ *Pro-glycative and pro-inflammatory activity*

AGEs seem to have a key role in cardiovascular disease and diabetes complications where hyperglycaemia heightens the glycation process of proteins in red cells, endothelium , eye , kidney tissues. In cardiovascular diseases, AGEs may bind collagen in blood vessels by the glycation of free amino acids (Yamagishi, 2011). The phenomenon can occur in diabetic people eating food rich in AGEs and cause arterial stiffening, myocardial abnormalities and contribute to atherosclerotic plaque (Vlassara & Palace, 2002).Bucala (1994)discovered that after glycation, LDL cholesterol crosslinks in blood vessel and it´s accumulated in arterial walls. Afterwards, macrophages are attracted and followed by foam cell; sub-sequentially the atheroma plaque appears. Among advanced MRPs: pentosidine, pyrraline and N^{e}-carboxymethyl-lysine CML have been the most studied in terms of food source extraction, metabolic absorption, transit, and recovery during elimination in urine and faeces (Delgado-Andrade, Tessier, Niquet-Leridon, Seiquer, & Navarro, 2012; Foerster & Henle, 2003; Sugiyama et al., 1998).

Moreover, some interesting studies contemplate AGEs involvement in the pathogenesis of:

- Osteoporosis; AGEs might affect bone and cartilage pathologies like arthritis rheumatoid through autoimmune response alteration. In other studies, AGEs could remodel impair bones and contribute to the osteoporosis phenomenon. CML has been found in high level in serum of patients with the disorder and a general skeletal fragility was measured in subject with AGE accumulation in the bones (Dong, Qin, Xu, & Wang, 2011; Hein, 2006; Neumann et al., 2014; Vashishth et al., 2001).

- Neurodegenerative disorders as Alzheimer and Parkinson; AGEs generate a loop of auto formation and hyper-phosphorylation of tau protein constituent in neurofibrillary tangles (marker of Alzehimer). Also in Parkinson, AGEs and RAGEs presence in the frontal cortex appear involved in aggregation of proteins which cause the disorder(Castellani, Smith, Richey, & Perry, 1996; Pamplona et al., 2005;V. P. Reddy, Obrenovich, Atwood, Perry, & Smith, 2002).

- Sarcopenia; the loss in muscular straight and mass called under the name of sarcopenia could also be connected to AGEs promotional action on aging, oxidative stress and inflammation (de la Maza et al., 2008; Haus, Carrithers, Trappe, & Trappe, 2007).

AGEs detection methods: history, limitations and state of art

AGEs quantification can be accomplished through either immunochemical or instrumental methods, including HPLC/ LC and GC coupled to MS(Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Gadgil, Bondarenko, Treuheit, & Ren, 2007; Münch et al., 1997; Zhang, Huang, Xiao, & Mitchell, 2011). The immunochemical methodis basicallyenzyme–linked immunosorbent assay:ELISA (Uribarri et al., 2005).Especially used in the past until recent years, it turned out to be unspecific, because it overestimates the AGEs content, since it is a fluorescent method that detects all the brownish compounds, so many interfering elements can false the analysis, furthermore it relates to a general sum of structures without targeting each representative AGEs analyte(Davidek, Clety, Devaud, Robert, & Blank, 2003).ELISA doesn´t give satisfying results even for the matrix effect that could mislead the detection of hundreths-fold compared with chromatography associated to mass spectrometerbased techniques, where mass transition, retention time and ionization in parent fragments represents a more suitable system, even though the AGEs wide range of polarities and

chemical characteristics makes it challenging and kits are not available yet(Assar, Moloney, Lima, Magee, & Ames, 2009; Henle, 2003; Uribarri et al., 2010). Nowadays, the limit is that generally just one or two markers are measured. Recovery experiments, validation methods on standards and proper calibration curves are lacking, therefore it is needed to extend the span of AGEs to cover all the chain of MRPs and obtain a more accurate implementation. The treatment of the samples is another concern. Hydrolysis access free AGEs prior chromatography is required, thuspeptide bonds must be broken down. Two ways exist but both of them present disadvantage and advantages, hence the achievement of a realistic quantification is difficult. Acid hydrolysis is the most effective, but it destroys AGEs which are not stable to strong acid (usually 6M HCl), although it works perfectly for acid resistant compounds like CML, and crosslinked AGEs able to tolerate these harsh conditions (Delatour et al., 2009). The alternative enzymatic hydrolysis, on the other hand, does nothydrolyse all peptide bounds and the result is a mixture of peptides and amino acids, with and without AGE modifications.HMWs AGEs should be isolated from sample before acid hydrolysis and proteins precipitated through 20% thricloroacetic acid, ultrafiltration and Folch extraction. As already anticipated above, this method is easier and cheaper than enzymatic digestion but itdegrades acid unstable AGEs (MGH1 derived), hydroimidazolones and pyrraline(Ames et al.; 2008). In this case, enzymatic digestion can be the solution and should be done using endo- and exo- peptidases combined in a cocktail-proteinase K, pronase E, peptidase, aminopeptidase, carboxypeptidase Y provide the best AGEs yield(-Glomb & Pfahler, 2001), but the disadvantage here is the removal of AGEs crosslinks and the unfinished chopping that falseAGEs amount. For all these reasons, the acid stable CEL, CML and pentosidineare generally employed as markers(Thornalley et al., 2003).

AGEs remarkable animal and clinical studies: A quick look at what has been done in the last 4 decades

A discrimination between AGEs dietary sources and *in situ*body reaction productsmust be considered. Indeed, at 37 °C -the set temperature in biological systems-Maillard reactions can easily take place over long time and when formed *in vivo*, they accumulate in some tissues or are present in the systemic circulation playing a fundamental role in metabolic and aging disorders (Knecht et al., 1991; Sharp, Rainbow, & Mukherjee, 2003). During the last 3 decades,AGEs

accumulation *in vivo* has been suggested to initiate insulin resistance because of decreasing bioactivity of insulin, inhibition of insulin secretion, and insulin signalling in skeletal muscle, adipose tissue, and liver(Jakuš & Rietbrock, 2004; Karachalias, Babaei-Jadidi, Ahmed, & Thornalley, 2003; Šebeková & Somoza, 2007; Vlassara & Striker, 2016; Yue et al., 2015).Several animal models have been adopted to elucidate the mechanisms that connect the intake of AGEs to physiological systems(Singh, Bali, Singh, & Jaggi, 2014). For instance, after feeding rats with a boiled bases and low-AGEs containing diet, the reduction in the serum of AGE levels was correlated with the improvement of different pathological biomarkers for cardiovascular risk and metabolic syndrome. The effects were particularly evident in diabetic and hyper-cholesterolemic animal models (Teillet et al., 2000). In long-term animal studies, high AGE diets increased renal dysfunction, oxidative stress, insulin resistance and reduced lifespan compared to the low AGEs(Uribarri, Cai, et al., 2007; Uribarri, Stirban, et al., 2007).In another study, the increase of the CML concentration in the tail tendon of healthy animals fed with a high AGE diet was associated with vessels stiffness, implying a possible reduction in joint mobility(Roca, Grossin, Chassagne, Puisieux, & Boulanger, 2014). CML presence has been confirmed in tissues such as cardiac muscle of rats fed with AGEs derived from bread crust and aortic rings of mice fed with CML-enriched diets(Roncero-Ramos et al., 2013; Roncero-Ramos et al., 2014). An association with the diet was proposed, and a direct relationship between CML and its deposit in organs was recently established by Tessier et al. (2016) using radio-labeled CML, but to what extent AGE accumulation in tissues could have cause-effect pathological consequences is not clear.Oppositely, trials that used a single AGE-rich ingredient (not boiled versus roasted diet) led to the conclusion that AGEs do not pose any risk to health (Ames, 2007).Scepticism about the negative impact of AGEs is justified by scarce knowledge about mechanisms underpinning the claimed effects. This was particularly the case of the work performed feeding rats with glycated casein and glycated soy protein, where no significative inflammation or oxidative stress was found in mice serum(Chuyen, Arai, Nakanishi, & Utsunomiya, 2005). However,despite animal assays have indicated a significant impact of high AGEs intake on metabolism and chronic diseases, it is difficult to extrapolate information about human riskbecause rodents unlike humans are not use to eat heated food (Tessier &Birlouez-Aragon 2012; Sebekova et al., 2005). In respect of clinical biomarkers, in many human studies,AGEs ingestionhas

been correlated with oxidative stress, serum CRP concentration, endothelial dysfunction, and insulin resistance (Vlassara et al.,2002). In diabetic volunteers it has been proved that along with glycoxidation of LDLs (Bucala et al. 1994), AGEs are suspected to bind and transform LDLs after absorption (Cai et al., 2008) promoting their intracellular oxidation and leading to vascular toxicity in AGEs based diet compared to low AGEs diet, despite comparable fasting glycaemia between the groups. The confirmation thatAGEs can provoke atherosclerosis in diabetic patients came fromNegrean et al. (2007)where in a single meal study, they found that low AGEs diet improved the postprandial micro- and macro-vascular functions. Again, the observational study by Uribarri et al. (2007) performed on healthy subjects showed a significative correlation between AGEs intake and a serum marker of inflammation; the high sensitivity C reactive protein (hsCRP). Besides, during interventional studies, the high- versus low-AGE approach has been implemented. Cooking the same food in different ways demonstrated alteration of risk factors in the roasted/fried high-AGE diet. Some few randomized controlled studies have been executed to examine the association between dietary MRPs and biomarkers of cardiovascular and diabetes risks in healthy volunteers or in patients with diabetes or renal disease (Hegab, Gibbons, Neyses, & Mamas, 2012; Nin et al., 2011; Semba, Bandinelli, Sun, Guralnik, & Ferrucci, 2009).

The first human intervention study was designed with a validated measure of the AGEs present in the diet was in the frame of the ICARE European Project (Birlouez-Aragon et al., 2010). The ICARE study investigated the impact on established parameters indicator of cardiovascular risk, diabetes, non-insulinemic impairment of high versus low AGEs diets in healthy young adults. Subjects consumed breakfast, lunch, and dinner at a canteen for 4 weeks, so caloric intake and food categories were strictly controlled. The food group that contributed most to AGE exposure was the cereal group, which consisted ofbread (27%), breakfast cereals (12%), and biscuits (17%). Grilled meat and fish contributed to only 16% of CML intake, which is similar to the percentage contributed by dairy products (14%). When a portion of 200 mL of UHT milk was added to the calculation the relative contribution of the dairy products to total CML intake becomes higher (19%) as reported by Tessier and Birlouez-Aragon (2012).

Levels of high density lipoprotein (HDL), total cholesterol, triglycerides, fast-

ing insulinemia, and HOMA were higher, while Vitamin E and C were lower after the high-AGE diet compared to the low-AGE diet and it was registered a correlation between CML and lipid fractions in plasma. For technical reasons such as the MRP dilution in food samples, the contribution of beverages was not measured in this part of the ICARE study. To take into account is that other MRPs such as the group of imidazolones, which have been detected in beverages and foods and *in vivo*, were not quantified in the meals of the ICARE study although their reported high concentration in foods. The authors of the ICARE study theorized that plasma CML does not come from diet but is rather produced by the *in vivo* reaction between proteins and reactive carbonyl compounds originated by the oxidative stress. The main un-controlled factor in the ICARE study was the gap in average energy density between the high- and low-AGE diets (2.1 versus 1.7). This is basically almost inevitable as strong thermal stress determines evaporation of water from food and an higher concentration of a 25% in calorie density that might be responsible for most of the observed effects. It is worth mentioning that the individuals in the high-AGE group ate 10% more calories than those in the low-AGE group, giving credit to the idea that the caloric restriction, was the main cause of beneficial health effect (Spindler&Dhahbi 2007). A second intervention study was performed on overweight Danish women (Mark et al., 2014). Participants were trained to follow either a high-AGE or low-AGE diet for one month, and the concentration of AGEs was measured on standardized meals. Results remained consistent with the ICARE study: the low-AGE diet decreased urinary AGEs, fasting plasma insulin concentrations, and homeostasis model assessment of insulin resistance (HOMA-IR). Nevertheless, in this case thefatintakewassignificantlyreduced(22%less)inthe low-AGE diet.A third study, which was also on overweight subjects, was performed in Australia (De Courten et al.,2016). In order to control the AGE intake, all meals were precooked and provided to participants for their home use. A crossover study with a relatively short intervention time (two weeks) was designed. Results clearly showed that low AGE intake increased insulin sensitivity. However, there were no significant changes in serum AGE concentrations. De Courten and co-workers (2016) suggested that restriction of dietary AGE content may be an effective strategy to decrease diabetes and CVD risks in overweight individuals.However, a stricter correlation with protein-bound serum CML than with free CML of dietary origin was recognized.On the other hand, observational studies concurrently showed that

the concentration of plasma CML is not a predictor of cardiovascular events or kidney failure in diabetic patients (Busch et al., 2006). Surprisingly, Sebekova et al. (2009) noted that the concentration of plasma AGEs is lower in obese children compared with thin control children.

AGEs and particularly CML are stable, unreactive compounds. One theory claims it is unlikely that AGEs can damage tissue by reacting directly with tissue proteins, but this action is well performed by dicarbonylcompounds *in vivo*, whose formation has been definitively linked to a high-fat diet intervention studies with roasted food(Xue et al., 2011; Sampath et al., 2017). In general, the contradictory findings could derive from observational/cross sectional wrong studies method to calculate AGEs concentration in food. It is based on the assessment through dietary record questionnaire, thus it´s not very reliable(Uribarri et al., 2003; Sebekova., et al 2008;Ubarri et al 2007; Dittrich et al.,2009).An un-expected chemopreventive behavior has been identified in an AGE called pronyl-lysine. Its induction *in vitro*of the anti-cancer liver phase II enzymes glutathione S-transferase (GST)is well described (Prochaska&Talalay 1988). Lindenmeier et al. (2002) illustrated that pronyl-lysine isolated from bread crust could prevent oxidative damage in human intestinal epithelial cells. They observed a significant increase of GST activity by 12% or 34% when exposing cells to synthetic pronyl-lysine products that could be englobed inside bread melanoidins. Tissue distribution and renal clearance of the AGE levels widely vary in healthy adults, suggesting that dietary AGEs may be detrimental only in combination with a co-existingdiseases.Aclearcorrelationhasbeenfound only for patients with renal impairment because of an inefficient excretion system (Kratochvilova et al. 2011). This increase was attributed to the lack of excretion of endogenous AGEs (Henle & Miyata 2003) and accordingly an intervention study based on a low AGEs diet in these patients showed a certain decrease of circulating AGEs (Vlassara et al., 2009).

However, the experimental designs are often deviated by many confounding factors like the un-calibratedcaloric density of the foodstuff. More trials testing the effects of a single AGE or of a AGE-rich ingredient added to the same food matrix are needed to demonstrate and assure the AGE physiological effects. Another problem is that the studies were assessed by the data measured using the unreliable ELISA method.Niquet-Léridon et al. (2015)compared ELISA and LC-MS/MS methods on CML measurement of some food categories

(fat, protein, and carbohydrate), and showed that LC-MS/MS method performs much better.deCourten et al.(2016) have recently showed the possibility that low-AGE diet increase insulin sensitivity in healthy overweight individuals. In this study, they measured dietary, plasma, and urinary AGEs contents, such as CML, CEL, and MG-H1, using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Another aspect to take into account is about the enzymatic ability of human digestive apparatus and the rate of absorption of HMW and LMW AGEs in the gastrointestinal tract, including peptide-bound forms and carbonyls that can really pass into the bloodstream and contribute to the body burden of AGEs. Meanwhile, it is still unfair for the currently insufficient evidence, to discourage AGEs ingestion in diet because research in this field is unripe, due to the fact that the clinical trials are short or include small sized sample of subjects (Clarke, Dordevic, Tan, Ryan, & Coughlan, 2016; Pouillart et al., 2008). In the future, digestion, absorption, behavior and elimination of dietary AGEs in human body must be explored through sophisticated and accurate tools and more effort should be engaged on caloric balance to make low and high AGEs diet more comparable (Kellow & Savige, 2013). A database of AGEs food fingerprints formulated on HPLC/LC –MS analysis would be a crucial step to offerfuture advancement on health implication.

Future perspective of MRPs research and potential value of beer matrix analysis

Scientific society argues about the un-healthy effect of beer, focusing on the“detrimental” presence of ethanol -which can beregulated since this target is easily achieved in low and free alcohol beer.We must not forget, instead, thesubstantial contribution of several other bioactive compounds worth an attempt of research. In fact,little amount of alcohol, in combination with anti- oxidants or anti-inflammatory substances can turninto a positive synergy (Peng, Ma, Chen, & Wang, 2011).AGEs or melanoidins quantification and characterization in brewing, for instance, could clarify Maillard reaction products evolution profile on a whole industrial process from malt, wort, up to fermented beer. Furthermore, the use of varying degrees kilned and roasted malt,derived from different thermal techniques, offers the chance to originate a large span of MRPs. This is testifiedby the broad range of colour and aroma emanation from pale to caramel, until super dark shades or from fruity to nutty flavours. Both melanoidins

and AGEs are present in this complex matrix. However, only one remarkable *in vitro* study tried to correlate the promising melanoidins content in different styles of beer to health impact, in particular to the suspected ACE-inhibitory activity. The results displayed that stout beers had higher hypotensive power, whereas no significant differences between light beer melanoidins and abbey type oneswere found. As previously stated for coffees, the stronger the heat treatment, the higher the ACE-inhibitory activity of beer melanoidins is.(Rufián-Henares & Morales, 2007).The authors concluded that ACE-inhibitory activity was also partly due to the low-molecular-weight compound non chemically bound to the melanoidins structure, such as the famous class of polyphenols in coffee named chlorogenic acids; in this contest melanoidins can act as carrier-protecting agents and in beer they can be coupled to polyphenols coming from hops. Xanthohumol is very abundant and seems to be protective against DNA damage(Ferk et al., 2010; Ho, Liu, Chen, Duan, & Lin, 2008), but many other chemical structures existent in a vegetal source like beer may contribute as well. Flavonoids are well known antioxidants that could trap intermediates of AGEs and decrease the final level in food and serum after digestion; this provides one more reason to dig into beer matrix (Wu & Yen, 2005).A later option, would be to track the metabolic transformation when beer MRPs cross the gastrointestinal barrier and check how much reach the bloodstream influencing some pathological risk factors. Recently, as stated above, the presence of AGEs in beer has been revealed through more advanced techniques as HPLC and LC-MS but there are just two papers confined to the analysis of final commercial beer.Current acquirements about AGEs determination in beer arrived thanks to HPLC-tandem MS method from a German study, where seven Maillard reaction products (MRPs): fructosyllysine, maltulosyllysine, pyrraline, formyline, maltosine, MG-H1, and argpyrimidine, were synthesized and quantitated in different types of beer (pilsner, dark, bock, wheat, and non-alcoholic beers) via application of the standard addition method. Free MRPs were analyzed directly. A high molecular weight fraction was isolated by dialysis and hydrolyzed enzymatically prior to analysis. Maltulosyllysine was quantitated for the first time in food. The most important free MRPs in beer were fructosyllysine (6.8–27.0 mg/L) and maltulosyllysine (3.7–21.8 mg/L). Beer contains comparatively high amounts of late-stage free MRPs such as pyrraline (0.2–1.6 mg/L) and MG-H1 (0.3–2.5 mg/L). Minor amounts of formyline (4–230 µg/L), maltosine (6–56 µg/L), and argpyrimidine (0.1–4.1 µg/L) were detected. Maltulosyllysine was

the most significant protein-bound MRP as maltose is one of the major sugars produced during mashing from starch degradation. Protein-bound and free MRPs exhibited different ratio, which suggests the influence of biochemical and microbiological parameter during the brewing process(Hellwig, Witte, & Henle, 2016). Therefore, in the future more research should be implemented to understand MRPs genesis from raw material down to final product.In a recent Japanese article, the determination of AGEs was discussed in beer and beer-typed liquor (Nomi et al., 2016). In beer samples, CML, CEL, and MG-H1 could be detected and quantitated for the first time via LC-MS. The levels of these AGEs were in the range of 0.07−0.24 μg/mL for CML, 0.07− 0.14 μg/mL for CEL, and 0.09−2.24 μg/mL for MG-H1. Previously, Glomb, Rösch, and Nagaraj (2001)isolated argpyrimidine as a methylglyoxal-arginine modification compound, and identified and determined N-carboxymethylproline (CMP) and N-formylproline (FP) as novel proline derived AGEs. Rakete et al. (2014)reported that CMP and FP were contained in the range of 0.21−0.59 μM and 1.8− 4.4 μM in beer. However, they could not detect CML because of the low lysine concentrations in beer. In spite of these backgrounds, Nomi et al. could determine more accurately than in the past CML, CEL, and MG-H1 in beer and showed the efficiency of the developed method.

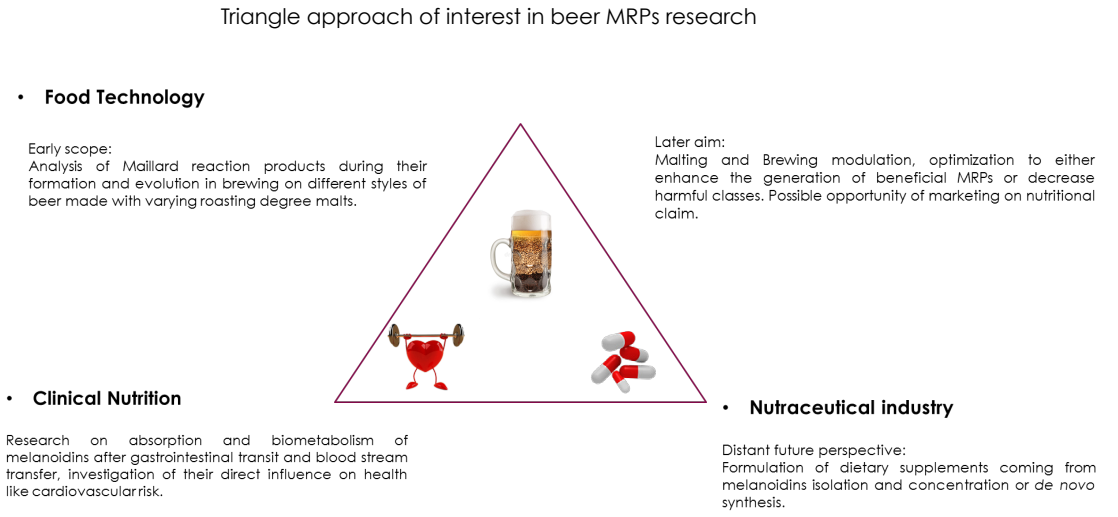

Figure 3. Triangle multidisciplinary approach on Maillard reaction products from beer. A branching concept can be unravelled from Malting and Brewing Science where Food Technology interest, Clinical Nutrition hope and Nutraceutical companies aim meet in the common purpose to offer a safer product.

Notably, research about Maillard reaction products in beer, embraces a multi-disciplinary approach that goes beyond Brewing Technology interest. The three fields touched would be: Food Technology, Clinical Nutrition and Nutraceutical Industry as illustrated figure 3. Infact, the discovery of how optimize the mashing, boiling, fermentation system, the choice of recipes oriented toward a reduction of harmful AGEs or all the way around, the increase of beneficial MRPs, may lead to the production of an healthier beer, while at the same time, the breweries could exploit opportunity of marketing with nutritional claim. On the other hand, their flow through the human metabolism, the real rate of absorption and bio-transformation could reveal interesting effect on cardiovascular risk leading toconclusions that until now have been just speculated. Beer as recreational beverage is consumed not only occasionally but very often and appreciated especially among young and adult people that see beer like an element of gathering. Public health could benefit from this informationto better explain the nature and role of Maillard reaction products in diet and their impact on humans, implementing the same research on clusters of food, in order to suggest nutritional guidelines to populations. Last but not least, the distant future perspective may open new horizon for beer, or also malt and hops separately: giving access to nutraceutical industry with the formulation of dietary supplements. Indeed, AGEs inhibitors from natural products with relatively low toxicity are promising candidates for the development of functional additives and even drugs for the treatment of diabetic complications and other AGE-associated diseases. Besides, more research must be carried out on the promising final melanoidins. In conclusion, there are no doubts about how attractive beer can be not only for the mixture of MRPs present but even because of the complementary or hampering presence of other bioactive compounds that integrate the overall behaviour and can alter, modulate or amplify the original isolated action of each MRP.

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