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MAILLARD REACTION PRODUCTS: OCCURRENCE, MITIGATION STRATEGIES AND THEIR PHYSIOLOGICAL RELEVANCE

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1. General introduction

After more than 100 years from the first paper describing the reaction between sugar and amino group leading to browning formation and later on indicated as Maillard Reaction is still at the very centre of the interest of scientists of different disciplines (Maillard, 1912). Among food scientists Maillard Reaction (MR) is important because of colour and flavour formation in an enormous variety of processed foods; while in medical science many of the complications caused by the undesired glycation of proteins in the presence of an excess of sugar, i.e. in diabetic conditions, arose from the formation of MR products, medical doctors refer to it as protein glycation, which impaired the functionalities of proteins.

Such an interest devoted to a single chemical reaction might appear weird at a first glance; but actually the reaction between carbohydrate and proteins is the cornerstone of many of the most important reactions taking place in food during processing because it is the origin of the colour, flavour, texture and taste of many heat treated products. This was exactly what Louis Camille Maillard anticipated when he discovered the reaction envisaging that it could play a fundamental role in many different research fields, food chemistry, food technology, fundamental biology, diabetics, eye health and nutritional science.

1.1 Description of the Maillard Reaction

The application of heat during industrial processing and household cooking of foodstuffs encompasses a variety of processes, such as boiling, frying, steaming, baking, stewing and roasting, in traditional and microwave and steam ovens. Industrial thermal treatment of foodstuffs includes many of the processes also listed for household cooking. In addition, heat has been used in traditional transformation processes other than cooking, such as toasting, kilning coffee roasting, drying processes, canning, pasteurization and related technology (UHT treatment) smoking and extrusion cooking. It is important to note that these processes can be controlled much better on industrial scale than on household level.

The quality of food, from the nutritional, microbial safety point of view and sensory aspects depends on a range of variables from farm to fork, including the quality of the raw material, processing techniques, packaging and cooking. The main purpose of industrial food processing is to provide safe and high quality food as demanded by the consumer (Moskowitz *et al*., 2009, Luning and Marcelis, 2009). The conduction of thermal processing in an appropriate way is the key to obtain safe food and in many cases also with enhanced nutritional functionality respect to the starting raw material (Van Boekel *et al.*, 2010).

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1.2 **Relevance in different foods**

Thermal processes are frequently used in food manufacturing to obtain safe products with a prolonged shelf-life and may have a strong impact on the final quality of foods. Baking, toasting, frying, roasting, sterilization etc. result in desired and undesired effects which all stem from the chemical reactions, namely Maillard reaction (MR), caramelisation and to a minor extent, lipid oxidation, occurring while the foods are heated. One of the purposes of thermal processes is to alter the sensory properties of foods, to improve palatability and to extend the range of colours, tastes, aromas and textures in foods produced from similar raw materials. Heating also destroys enzymes and micro-organisms and lowers the water activity of the food to some extent thereby preserving the foods.

In the Figure 1 the progressing of Maillard reaction in different foods is illustrated highlighting how the decrease of water activity and the increase of thermal load parallel the development of browning in different foods.

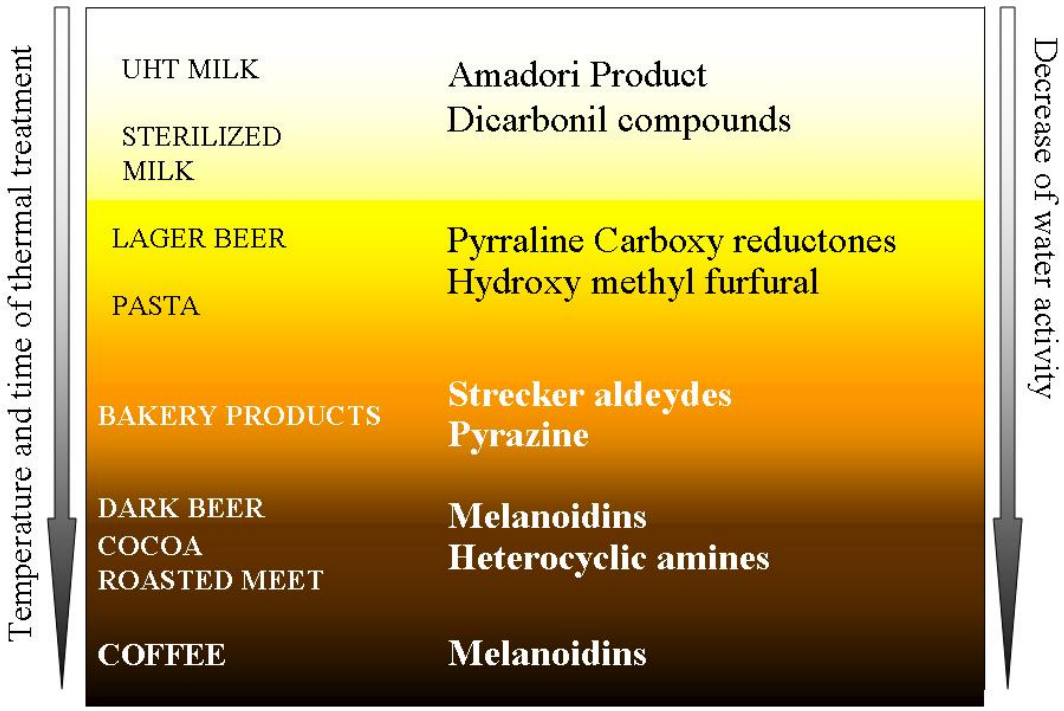

Figure 1: List of different foods which undergo Maillard Reaction during thermal treatment (Anese and Fogliano 2001). Colours remind those formed in the foods. In the second column the main Maillard reaction products formed along the different stage of the reaction development are highlighted.

In milk and dairy products MR is almost always undesired with the exception of some desserts like the “dulche de leche” or the Norwegian brown cheese. In dry pasta the accurate control on MR

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during drying allowed to obtain dried pasta with better technological features (less starch leaching during cooking), better visual appearance (golden yellow colour) and improved storage performance. In all bakery products MR products are the main determinants of sensory quality influencing color, flavour and texture. Moving on towards the seeds thermal treatments because of the reduced amount of water the MR rapidly produce a dark colour and great amount of melanoidins. In particular coffee represent one the most important contributors for the dietary intake of Maillard reaction products and it has been estimated that up to two grams per day of melanoidins can come from coffee brews.

1.3 Why is still necessary to study this reaction

The Maillard reaction is, together with fat oxidation, the chemical phenomenon that contributed to the quality of processed foods. Therefore the efforts for its understanding and control are of utmost importance to support the development of food industries. Beside the relevance in the applicative fields of food science a deep understanding of the reaction mechanisms are essential for fundamental biological issue such as those related to the ingestion of these products. The issue of the dietary AGEs (which is basically a different way to name the MR products) comes up in the last years as a possible cause of dietary related disease. Some studies demonstrating the interaction of these AGEs with endogenous receptor (named AGE receptors - RAGE) which is in turn able to trigger biochemical pathways underpinning inflammation and oxidative stress. There is a lot of debate about the relevance of dietary AGEs in this process which also occurring with endogenously formed AGEs. In vivo human trials measuring a number of physiological parameters after consumption of severely heated foods gave contradictory results: however they concur in showing some risks associated to dietary AGEs consumption for specific categories of patients such as those suffering of renal diseases, uremic and diabetics.

One of the main weaknesses in this study is the characterization of the MR products present in the foods and so their effective dietary intake, the same problem which is found in all dietary questionnaires used to evaluate the dietary intake in population studies.

In the last years the issue of the potential risk linked to the intake of food containing MR products was mainly related to the acrylamide formation which will be explained in the following paragraphs.

1.4 Potentially hazardous compounds the case of acrylamide

Beside to these positive effects, some detrimental consequences of thermal processes must be carefully evaluated. The loss of thermo labile compounds such as vitamins as well as essential

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amino acids (lysine, triptophane) and/or the formation of undesired tastes and off-flavours are well established phenomena bringing about a loss in the nutritional value and sensorial quality of heated foods. All the same, the major concern arising from heating processes come from the formation of hazardous compounds, the so-called food-borne toxicants i.e. compounds that are not naturally present in foods, but that may be developed during heating or preservation and that reveal harmful effects such as mutagenic, carcinogenic and citotoxic effects. Well known examples of these food-borne toxicants are heterocyclic ammines, nitrosamines and polycyclic aromatic hydrocarbons. Recently two food-borne toxicants have gained much interest because of their high toxicological potential and their wide occurrence in foods: Acrylamide and hydroxymethylfurfural (HMF). Acrylamide has been added to the list of food-borne toxicants since in 2002 Swedish National Food Administration found out relevant amount of acrylamide in several heat treated, carbohydrate-rich foods such as potato chips and crisps, coffee and bread (Swedish National Food Administration, 2002).

Shortly after its discovery in foods, it has been clearly established that the major pathway for acrylamide formation in foods is Maillard reaction with free asparagine as main precursor (Mottram, Wedzicha and Dodson, 2002; Stadler *et al.*, 2002; Zyzak *et al.*, 2003; Stadler *et al.*, 2004). Asparagine can thermally decomposes by deamination and decarboxylation but when a carbonyl source is present the yield of acrylamide from asparagine is much higher explaining the high concentration of acrylamide detected in foods rich in reducing sugars and free asparagine such as fried potatoes and bakery products (Mottram, *et al.*, 2002; Yaylayan, *et al.*, 2003; Weisshaar and Gutsche, 2002). Other minor reaction routes for acrylamide formation in foods have been postulated, from acrolein (Yasuhara, *et al.*, 2003), from acrylic acid (Yasuhara, *et al.*, 2003) and from wheat gluten (Claus, *et al*., 2006). Finally, acrylamide can be generated by deamination of 3-aminopropionamide (3-APA) (Granvogl and Schiberle, 2006). 3-APA is an intermediate in MR, can also form by enzymatic decarboxylation of free asparagine and can yield acrylamide upon heating even in absence of a carbonyl source (Granvogl, *et al*., 2004; Granvogl and Schiberle, 2007).

1.4.1 Occurrence in foods and dietary exposure

Since 2003, data on the occurrence of acrylamide in food commodities have been submitted to the Joint Research Centre (JCR) of the European commission by member states both from competent authorities and from food industry. In April 2009, the European Food Safety Agency (EFSA) (EFSA, 2009) reported the results of the monitoring of acrylamide levels in foods in response to a request of the European commission (commission recommendation 2007/331/EC) (European Commission, 2007). Data collected in this report concern with foods sampled in 2007 and

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submitted to the commission by 21 member states and Norway. Two additional reports based on food sampled in 2008 and 2009 are anticipated to be published by EFSA in the next two years on yearly basis. A summary of the results recently reported by EFSA is given in table 1

Table 1 Acrylamide levels (μg/kg) in different food commodities (EFSA, 2009).

Food commodities	N*	Median	Mean	Maximum
Biscuits	227	169	317	4200
Bread	272	50	136	2430
Breakfast cereal	128	100	156	1600
Cereal-based baby foods	76	42	74	353
Coffee	208	188	253	1158
French fries	529	253	350	2668
Jarred baby foods	84	31	44	162
Other products	854	169	313	4700
Potato crisps	216	490	628	4180
Home-cook potato	121	150	319	2175

*Number of individual data analyzed for each food category.

Acrylamide is formed during frying, roasting, and baking and is not typically found in boiled or microwaved foods. The highest acrylamide levels have been found in fried potato products, bread and bakery wares, and coffee. Acrylamide has been found, however, in food products other than those listed in table 1 such as hazelnuts and almonds (Amrein, *et al.*, 2005), olives and recently, in foods not subjected to severe heating, such as dried fruits (e.g. plums, pears, apricots) (Amrein, et al., 2007). Although acrylamide concentration in such products can be very high, their contribution to the overall acrylamide intake is marginal. Animal derived heat treated foods such as meat and fish, generally exhibit low or negligible levels of acrylamide (Swedish National Food Administration, 2002; EFSA, 2009; European Commission, 2006). All the same, a great variability in acrylamide level between different products of each food category as well as between different brands of the same product has been reported. The difference in the concentration of precursors (free asparagine and reducing sugars) in raw materials, difference in food composition and in process conditions applied can easily explain the observed variability (Boon, de Mul, van der Voet, van Donkersgoed, Brette and van Klaveren, 2005). Moreover, the actual acrylamide content of a food as it is eaten can largely vary according to domestic cooking conditions.

Estimates of dietary acrylamide intake have been made for populations in many countries. A great variability between populations has been found according to population's eating habits and the way the foods are processed and prepared. Dybing, *et al.* (2005) reported an average daily intake for adults close to 0.5 μg/kg body wt, with 95th percentile values of about 1 μg/kg body wt. The World

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Health Organization estimates a daily dietary intake of acrylamide in the range of 0.3–2.0 μg/kg body wt for the general population and up to 5.1 μg/kg body wt for the 99th-percentile consumers (WHO, 2005). The daily intakes of dietary acrylamide for the general population and high consumers (including children) are estimated to be on average 1 and 4 μg/kg body wt, respectively. Children eat more acrylamide than adults probably because of their higher caloric intake relative to body weight as well as their higher consumption of certain acrylamide-rich foods, such as French fries and potato crisps (Dybing, *et al.*, 2005). Heudorf, *et al.*, (2009) assessed the dietary exposure to acrylamide in 5-6 years aged children by means of urinary excretion of mercapturic acids as biomarker. They reported a median (95th percentile) daily uptake of acrylamide in children of 0.54 (1.91) μg/kg body wt thus confirming that children are a vulnerable subgroup of population. Arribas-Lorenzo and Morales (2009) estimated the dietary exposure from potato chips in the Spanish population. The authors reported a daily dietary exposure (based on a 3-days food record) from potato crisps of 0.053 μg/kg body wt for the adult population (17-60 years) and of 0.142 μg/kg body wt for children (7-12 years). In most of the populations, the major contributors to acrylamide intake are potato crisps and chips, bread and coffee each accounting for nearly one third of the total intake (WHO, 2005). Other food products can account for up to 10% of the total intake of acrylamide. However, some concern on the reliability of the dietary exposure assessments has been expressed. It is likely that consumption data rather than the chemical analysis mostly contribute to the uncertainties as the analytical methods for the quantification of acrylamide in potato and bakery products are well validated (Eriksson and Karlsson, 2005; Wenzl, *et al.*, 2007). Recently, two European reference materials (ERM) for the determination of acrylamide in food (crisp bread, ERM-BD272, and rusk, ERM-BD274) have been developed (Koch, *et al.*, 2009). These reference materials could be used to improve or validate the current, available analytical methods for bakery products. However, it would be valuable that additional reference materials could be released in the future for potato and/or coffee products.

On the other hand, several factors may complicate the precision in estimation of the actual dietary exposure to acrylamide. As stated above, the acrylamide levels of commercially available foods largely vary between brands and between production batches. In addition, storage duration and conditions as well as domestic cooking or catering conditions might strongly affect the actual acrylamide level of foods as they are eaten. Food frequency questionnaires (FFQ) based methods for the estimation of dietary acrylamide intake are usually not designed for the accurate assessment of the actual acrylamide exposure. Previous studies showed that food frequency questionnaires (FFQ) that are not designed to assess acrylamide dietary intake did not correlate with acrylamide-Hb adducts and the concentration of urinary acrylamide metabolites (Wilson, *et al.*, 2008; Hagmar,

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et al., 2005; Bjellas, *et al.*, 2007). Nevertheless, in 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that "exposure estimates are consistent and that new calculations or product analyses are not likely to change exposure estimates" (WHO, 2005). This conclusion has been recently reaffirmed by the expert panel of EFSA in the scientific colloquium on acrylamide on May 2008 for which "the analytical methods for establishing occurrence data and estimating human exposure appear adequate and, even with better data, it seems unlikely that the currently estimated margins of exposure will change dramatically" (EFSA, 2008).

1.5 Potentially hazardous compounds the case of HMF

5-hydroxymethylfurfural (=5-(hydroxymethyl)furan-2-carbaldehyde, HMF) is a furanic compound which forms as an intermediate in the Maillard Reaction (MR) (Ames, 1992) and from direct dehydration of sugars under acidic conditions (caramelisation) during thermal treatments applied to foods (Kroh, 1994). 3-deoxyosone is known as the key intermediate in HMF formation. It stems from 1,2 enolisation and dehydration of glucose or fructose. The further dehydration and cyclization of 3-deoxyosone yields 5-hydroxymethylfurfural.. In acidic conditions, HMF can form even at low temperatures (Lee and Nagy, 1990), although its concentrations, drastically increases as temperatures of thermal treatments or storage increase. Under dry and pyrolytic conditions an alternative pathway to HMF formation from fructose and sucrose has been proposed. It involves the formation of a highly reactive fructofuranosyl cation which can be effectively and directly converted to HMF (Perez-Locas and Yaylayan, 2008). HMF formation in foods can have different pathways. Apart from temperature, the rate of HMF formation in foods is dependent on the type of sugar (Lee and Nagy, 1990), on pH (Gökmen, *et al.*, 2007), on water activity (Kroh, 1994; Gökmen, *et al.*, 2008) and the concentration of divalent cations of the media (Gökmen and Şenyuva, 2006b).

1.5.1 Occurrence and dietary intake

The amount of HMF detectable in foods is directly related to the heat load applied during processing of carbohydrate-rich products. Another source of HMF is represented by ingredients used in the formulation such as caramel solutions or honey. HMF concentrations in foods can vary largely sometimes exceeding 1 g/kg in certain dried fruits and caramel products (Ibarz, *et al.*, 2000; Akkan, *et al.*, 2001; Rada-Mendoza, *et al.*, 2004). HMF could be also found in bakery products, malt, fruit juices, coffee, and vinegar. Generally, HMF could be used as marker of quality for a wide range of processed fruits (Rada-Mendoza, *et al.*, 2002; Rada-Mendoza, *et al.*, 2004), coffee (Dauberte, *et al.*, 1990) honey (Tosi, *et al.*, 2002; Fallico, *et al.*, 2004) and milk (Van Boekel, 1998; Morales and Jiménez-Pérez, 2001). HMF is also used for monitoring the heating processes applied

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to cereal products such as pasta drying (Resmini, *et al.*, 1993), bread baking (Ramírez-Jiménez, *et al.*, 2000a; Ramírez-Jiménez, *et al.*, 2000b), bread slices toasting (Ramírez-Jiménez, 1998) as well as extrusion of baby cereals (Fernández–Artigas, *et al.*, 1999; Ramírez-Jiménez, *et al.*, 2003) and breakfast cereals (García-Villanova, *et al.*, 1993). Although the concentrations in some food items such as dried fruits, caramel and vinegar are extremely high, bread and coffee are the most important contributors to dietary HMF intake (Murkovic and Pichler, 2006). The earlier estimates for HMF intake ranged from 30 to 150 mg/per person (Ulbricht *et al.*, 1984; Janzowski *et al.*, 2000). In a recent paper, Rufian-Henares and De la Cueva (2008) estimated the daily dietary intake of HMF in the Spanish population. Three different dietary exposure scenarios were depicted according to minimum, median and maximum value of analytical data on HMF concentration in foods. The authors reported a mean daily dietary intake of 2.1 mg, 9.7 mg and 23 mg for the three scenarios respectively. These results are in line with those reported by Delgado-Andrade, *et al.*, (2007) who calculated a daily HMF intake of 5.1 mg for Spanish adolescents as measured in the whole diet after a 24-h recall but are far below the estimates from Ulbricht, *et al.*, (1984). The authors suggested that the difference could be explained by an overestimation of HMF content of foods in the Ulbricht study due to the rough spectrophotometric methodologies used. In any case the estimated exposure is several orders of magnitude higher than the estimated daily intake for other heat-induced food toxicants such as acrylamide and furan (Morehouse, *et al.*, 2008). Recently, in a study on 53 Norwegian volunteers Husøy, *et al.*, (2008) estimated the HMF dietary intake by means of 24-hrs recall. The authors reported that the mean (95^{th} percentile) daily intake for HMF was 5.6 (27.6) and the amount of urinary HMFA (5-hydroxymethylfurfuroic acid, the major metabolite of HMF in humans) was 12.4 (28.6) mg/person respectively, thus suggesting alternative source to diet for HMF exposure. Pharmaceutical preparations and cigarette smoke should be considered as additional sources of exposure (Jellum *et al.*, 1973; Crump and Gardner, 1989). All the same, data on dietary exposure are very limited. Additional studies are therefore needed to assess average, medium and maximum intake for different populations and segments of population. For an accurate estimate of dietary intake, data on the concentrations of HMF in several foods when they are eaten are necessary. As highlighted for other food toxicants, domestic storage and cooking conditions may strongly affect the actual exposure to HMF.

1.6 Potentially health compounds: *N*-methylpyridinium; pronyl-lysine and melanoidins

The studies on the biological effects of compounds specifically formed during processing were traditionally focused on the potential harmful effects as they are considered somehow artificial. However the evidence of the beneficial effects exerted by some processed foods, such as coffee,

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promoted the studies aimed at investigating the mechanisms and the compounds responsible for the beneficial effects. Some small molecular weight compounds such as pronyl-lysine and *N*-methylpiridinium, as well as some quinide compounds derived from chlorogenic acids have been identified. Recently the structures of two classes of food melanoidins such as those present in coffee (Gniechwitz *et al.*, 2007 and Reichardt *et al.*, 2009; Bekedam *et al.*, 2007 and 2008) and in bread crust (Somoza 2005; Borrelli *et al.*, 2003) were elucidated allowing to shed some light on the possible biological activities of these compounds.

Next to bread crust and malt, coffee brews were also studied for their effects on Phase II enzymes (Somoza *et al.*, 2003). In this study, *N*-methylpyridinium iodide was identified as the key compound modulating Phase II GST. In vivo effects of a decaffeinated coffee beverage and *N*-methylpyridinium iodide were tested in a 15-day animal trial on rats. As a result, feeding of 4.5% coffee beverage resulted in an increase of Phase II GST and UDP-glucuronyl-transferase activity by 24% and 40%, respectively, compared to animals fed the control diet. Animals on the N-methylpyridinium diet showed an increase in liver Phase II UDP-glucuronyl-transferase of 65% compared to controls. The mechanism by which *N*-methylpyridinium ions induce Phase II enzymes was recently identified by Böttler *et al.*, (2009) who showed that this compound effectively induced the gene transcription and translocation of Nrf2, a major transcription factor leading to the expression of antioxidant and Phase II enzymes, such as GST. These results are in line with findings reported by Cavin *et al.*, (2008) who demonstrated an induction of Nrf2-mediated cellular defence and alteration of detoxifying enzyme activities as mechanisms of chemoprotective effects of coffee in the liver of rats. However, controlled intervention trials are still needed to verify the contribution of N-methylpyridium ions and potential other health beneficial compounds in coffee to the reduced risk for diseases such as various types of cancer which are associated with an endogenous load of reactive oxygen species and decreased activities of detoxification enzymes.

Melanoidins are widely distributed in thermally processed food and they are defined as polymeric high molecular weight, brown-coloured Maillard reaction end-products, containing nitrogen (Morales, 2002; Bekedam *et al.*, 2006) Their chemical structure is complex and still remains largely unknown (Kato, and Tsuchida, 1981; Hofmann 1998; Cammerer, and Kroh, 1995; Nunes and Coimbra, 2007). However, four main proposals of the structure have been put forward: (i) low-molecular weight coloured substances crosslinked to free amino groups of lysine or arginine in proteins (Hofmann, 1998) (ii) units of furan and/or pyrroles that, through polycondensation reactions, form melanoidins repeating units (Tressl *et al.*, 1998), (iii) skeleton mainly built up from sugar degradation products formed in the early stages of the MR, polymerized and linked by amino compounds, (iv) skeleton mainly built up form proteins crosslinked by Maillard reaction products

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(i.e. the melanoproteins) (Hofmann, *et al.*, 1999).

The absence of a known molecular structure and the strict dependence of its concentration on processing conditions in the final products have hampered an estimation of the dietary intake of melanoidins thus far. However, mounting evidence suggests that melanoidins are not an inert material and they can exert some physiological action.

The main sources of dietary melanoidins are definitely coffee and bakery products; however also other processed foods such as cocoa, malt, roasted barley, black beer, roasted potatoes, roasted pulses and seeds, meat, soy sauces, balsamic vinegar, sweet wine, processed tomatoes, contain melanoidins (Adams, *et al.*, 2005). Besides being the main dietary source coffee and bread melanoidins are also representative of the two main typologies of melanoidins. The principal constituent of coffee melanoidins is polysaccharides. However, in bread the main structure is a proteinaceous material and this melanoidins are referred as melanoproteins as well (Fogliano, *et al.*, 1999).

During roasting of coffee green beans chemical and structural changes taken place where polysaccharides, galactomannan-like and arabinogalactan-like carbohydrates, proteins, and phenolic compounds, mainly hydroxycinnamates, contribute to the formation of coffee melanoidins. D'Agostina, *et al.*, 2004; Illy and Viani, 1995). Recently, it was also demonstrated that phenolic compounds can also be non-covalently linked to coffee melanoidins and melanoidins could acts as carriers of low molecular weight substances.

In bakery products melanoidins are formed by gluten proteins cross-linked by coloured MR products, while other small molecular weight coloured MR products are entrapped in the gluten network. Melanoidins are present only in the crusts and can be considerably enhanced by the use of browning agent that can be added on the surface of the dough. Bread melanoidins concentration depends on the intensity of the thermal input: the higher the treatment the higher the concentration. Bread melanoidins are mainly water insoluble therefore they can be efficiently extracted only after extensive enzymatic digestion (Borrelli and Fogliano, 2005).

Melanoidins, and coffee melanoidins in particular, have different functional properties apart to their contribution to colour and technological properties, being able to bind flavours, exerting antioxidant capacity to foods and suppressing oxidative stress in cells, metal-chelating properties, antimicrobial activity, suppresses Helicobacter pylori adhesion, modulating chemopreventive enzymes, among others (Somoza, 2005). In the last ten years many studies suggested that they can have a relevant role in the gastrointestinal tract since melanoidins are fermented in the colon, and act as dietary fibre, modulating their bacterial population (Ames *et al.*, 1999; Dell'Aquila *et al.*, 2003; Gniechwitz *et al.*, 2008). It has been proposed that melanoidins should be considered as

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antioxidant dietary fibres which play a role in the prevention of cardiovascular disease and control of colorectal cancer. This statement is strengthened by the observation that a coffee melanoidins rich ingredient, the coffee silverskin, is able to promote Bifidobacteria growth, and by the fact that the degradability of carbohydrate part of the coffee brew melanoidins by human faecal microbiota was demonstrated (Borrelli *et al.*, 2004; Gniechwitz *et al.,* 2007). On the other hand it should be considered that most of the melanoidins are recovered in the faeces and that faecal antioxidant activity showed a direct correlation with coffee intake (Garsetti *et al.*, 2000).

1.7 Mitigation strategies to reduce acrylamide concentration in foods

A number of mitigation strategies to reduce the acrylamide content in foods have been proposed and tested up to now. They have been mainly focused on potato products and cereal-based products that are two of the major contributors to dietary exposure in most of the populations. Conversely, only very limited process options for coffee products are available for reducing acrylamide levels without affecting the final quality (CIAA, 2009; Guenther, *et al.,* 2007). The mitigation steps include changing in recipes and formulations (selection of potato varieties and cereal species or varieties low in acrylamide precursors, addition of proteins, glycine, cysteine and other aminoacids, organic acids and acidulants, calcium ions, cyclodextrin, natural antioxidants or antioxidant extracts etc., replacement of reducing sugars with sucrose and of ammonium bicarbonate with sodium bicarbonate) or changing in process conditions and/or technologies (Changing of time-temperature of frying or baking, changing in the type of oven, prolonged fermentation etc.). The removal of acrylamide after formation by means of vacuum has also been proposed but its impact on manufacturing practices and food quality has not yet been clearly established (Anese, Suman and Nicoli, 2009). The “toolbox” for acrylamide of the Confederation of the Food and Drink Industries of European Union (CIAA) collects most of the mitigation options that have been proposed so far (CIAA, 2009). The latest edition (updated at February 2009) includes information from food and beverage manufacturers in the USA, provided through the Grocery Manufacturers Association (GMA).

One the most promising tool to control acrylamide content in heat-treated foods is the addition of the enzyme asparaginase. The enzyme asparaginase (L-asparagine amidohydrolase), is an enzyme able to catalyse the hydrolysis of asparagine in aspartic acid and ammonia thus lowering the content of precursor asparagine. Asparaginase has been successfully applied at lab scale both to potato products (Zyzak *et al.*, 2003) and cereal-based products (Capuano, *et al.*, 2008; Capuano *et al.*, 2009; Vass *et al.*, 2004) with percentage of reduction up to 85-90% and no effect on products taste and appearance and is being already used for some products at industrial scale (CIAA, 2009).

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Some preliminary results achieved at lab scale highlight that asparaginase pretreatment of green beans may represent a viable way to reduce acrylamide concentration in roasted coffee as well. Up to now, two commercial products, Acrylaway® (asparaginase form *Aspergillus oryzae*) from Novozyme and PreventASe® (asparaginase form *A. niger*) from DSM, are on the market for food applications. GRAS status has been obtained from the US FDA for both types of asparaginases available and also JECFA endorsed the conclusion that asparaginase does not represent a hazard to human health (JECFA, 2007, JECFA, 2009). Nevertheless the high cost of the enzyme may represent a serious constraint on its application on a large scale.

It should be noted that most of the mitigation measures proposed so far were only tested at laboratory or at pilot scale. Therefore, for those mitigation measures it is not clearly known whether the percentage of reduction in acrylamide claimed at laboratory scale could ever be achievable in food processed at an industrial scale. When several mitigation strategies are applied to the same food product, the overall percentage of reduction in acrylamide level is not merely the sum of the percentages achievable when each single measure is applied. The interactions among different measures are not clearly known as well and should be taken into account.

It has been also emphasized that some mitigation strategies are associated with an increase in other risks or a loss in benefits. For example, prolonging yeast fermentation can efficiently reduce acrylamide concentration in bread but it is also associated to an increase in the levels of 3-monochloropropandiol (3-MCPD), another neo-formed contaminant (Hamlet and Sadd, 2005; Hamlet, *et al.,* 2005 and 2004). Similarly, replacement of ammonium bicarbonate with sodium bicarbonate as rising agent for fine bakery products results in an increase of sodium intake (Sacks *et al.,* 2001; Cook, *et al.,* 2007). There is a wide consensus that the actions aiming at lowering acrylamide content of foods should be accompanied by a risk-risk or risk-benefit analysis to elucidate all the side effects and their impact on human health. Several mitigation strategies for acrylamide reduction in bakery products are reported in the literature along with the concomitant effect on HMF concentration. It is obvious that some options are hardly feasible because of the negative effect they have on HMF content. In addition, some of the mitigation strategies that have been proposed bring about changes in organoleptic properties of foods (excessive browning as result of glycine addition, generation of off-flavours, insufficient browning as result of changing in time-temperature profile etc.) that can dramatically affect the final quality and consumers' acceptance (Hamlet *et al.,* 2005; Capuano *et al.,* 2009). This is a fundamental point for the future considering that mitigation strategies are completely not useful if for sensorial reasons consumers do not like the "mitigated" products giving their preference to the "conventional" ones. In that respect the knowledge of the kinetics of acrylamide accumulation in foods is of utmost importance.

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Acrylamide content of foods should be regarded as the result of concomitant reactions of formation and elimination. Acrylamide starts to form at a temperature>100°C after an initial lag phase during which no acrylamide forms. Later on, the acrylamide concentration increases exponentially with time to a maximum concentration, after which it can decrease again because of the exhaustion of one of the reactants and/or by the elimination of acrylamide. However, the mechanisms of elimination for acrylamide have not been fully elucidated and need further investigations. Acrylamide possesses two functional groups, an amide group and the electron-deficient vinylic double bond that makes it available for a wide range of reactions, including nucleophilic and Diel-Alder additions and radical reactions. Acrylamide may undergo Michael addition type reactions to the vinylic double bond with nucleophiles, including amino and thiol groups of amino acids and proteins. On the other hand, the amide group can undergo many reactions including hydrolysis, dehydration, alcoholysis and condensation with aldehydes (Friedman, 2003). Many kinetic models have been proposed to describe acrylamide formation and elimination and to predict its final concentration in model systems and foods. Single-response models based on empirical overall reaction kinetics have been extensively used. Acrylamide formation has been thus modelled as a first order reaction (Hedegaard, *et al.*, 2007; Gokmen and Senyuva, 2006a), as a pseudo-first order reaction (Amrein, *et al.*, 2006) or as a second order reaction (De Vleeschouwer, et al., 2006) according to reaction conditions and reactant concentrations. Acrylamide elimination has been usually modeled as a first order kinetics (Biedermann, *et al.*, 2002; Gokmen and Senyuva, 2006a). Totally empirical models such as those proposed by Corradini and Peleg (2006) and Kolek, *et al.*, (2007) have been also proposed and proved to satisfactorily describe acrylamide concentration in model systems (Zhang and Zhang, 2008) and potato chips (Knol, *et al.*, 2008). Such empirical models are not based on an underlying chemical mechanism and extrapolation is thus not possible outside the region of variables for which the function has been derived. On the other hand multiresponse models using acrylamide data supplemented with data on reaction precursors, intermediates and end products including mechanistic insights in the chemistry involved have been also proposed (Knol, et al., 2005; De Vleeschouwer, *et al.*, 2009a and 2009b), With such model systems not only acrylamide formation but also that of other relevant Maillard-related compounds can be modelled and the estimation of kinetic parameters is much more precise.

1.8 Mitigation strategies to reduce HMF concentration in foods

Up to now there are no available mitigation strategies specifically addressed to reduce HMF content in foods. The aim is challenging because of the nature of its precursors and formation pathways. HMF forms through Maillard reaction and caramelisation, which mostly contribute to desired

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colour, taste and aroma of heated foodstuffs. Unfortunately, HMF formation follows the same pathways leading to brown and flavour compounds. For instance, a high correlation between HMF content and browning development has been repeatedly reported (Capuano, *et al.*, 2008 and 2009) so that modelling the time-temperature profile by reducing heating times and/or temperatures is likely to reduce HMF concentrations in the same time resulting in a reduction of browning development which can potentially compromise the quality and acceptability of final products. The same happens when mitigation strategies based on changes in recipes are applied, for example by replacing reducing sugars with non reducing sugars or polyalchols. However, it has been also reported that replacing glucose or fructose with sucrose in biscuits results in higher level of HMF when baked at temperature>250°C (Ait-Ameur, *et al.,* 2007). A highly reactive fructofuranosyl cation has been postulated to form from sucrose at high temperature which yields HMF very rapidly (Perez-Locas and Yaylayan, 2008). As such, the kinetic de-coupling of the pathways yielding desiderate MR compounds and those yielding HMF is a very though goal to be accomplished. For example, knowledge of kinetics of HMF formation/elimination would be very useful in this respect. Studies on HMF formation are very limited and based mainly on empirical models describing HMF formation as a zero-order kinetics or as following an exponential trend (Capuano, *et al.*, 2008; Ait-Ameur, *et al.*, 2007; Gentry and Roberts, 2005; Chen, *et al.*, 2010). Mechanistic models (multiresponse modelling) have never been applied for this compound up to now. It would be a very valuable approach as multiresponse models enable the description of other (Maillard-related) reactions of importance such as colour and flavour development and the implementation of valuable optimization strategies. The effect of pH of the dough on HMF formation has been reported as well. Generally, increasing the pH of the dough results in a decreased level of HMF in bakery products (Gökmen, *et al.*, 2007). In fact, low pH as well as the presence of acidic catalyst promotes heat induced decomposition of furan derivatives (Lee and Nagy, 1990).

1.9 Encapsulation as innovative technologies to control MR development in foods

The complexity of MR is the major hamper to the development of mitigation strategies aiming at reducing the concentration of potentially harmful MRPs in food (Capuano and Fogliano, 2011).

The existing approaches, which are summarized in Figure 2 represented the starting point for the evaluation of the encapsulation as a innovative way to reduce the formation of MRPs.

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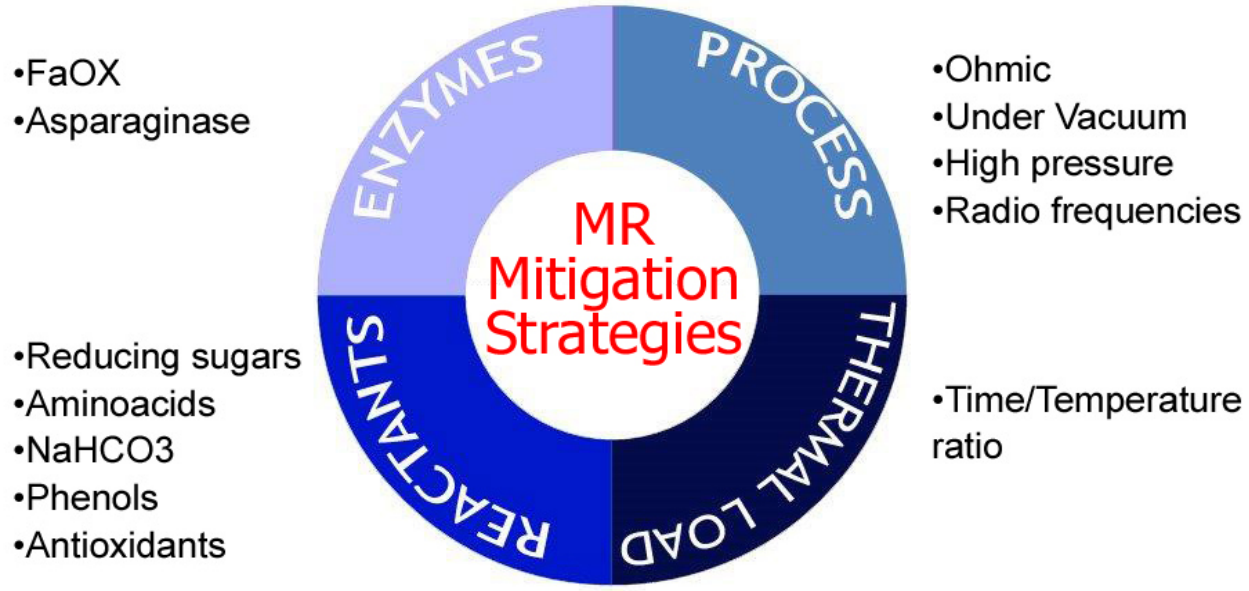

Figure 2 Starting point for the evaluation of the encapsulation as a innovative way to reduce the formation of MRPs.

In the recent years some works dealing with the application of encapsulation to control chemical reactions with different purposes have been published, however encapsulation applications to MR were not yet explored. In this paragraph the possibility to use encapsulation to subtract from the reaction environment some molecules playing a key role in the formation of MRPs will be discussed. The development of MR is determined by several factors beside the simple concentration of sugars and amino acids: some of these molecules are circled in the Figure 3 and can give an immediate idea about the potential strategies for food encapsulation.

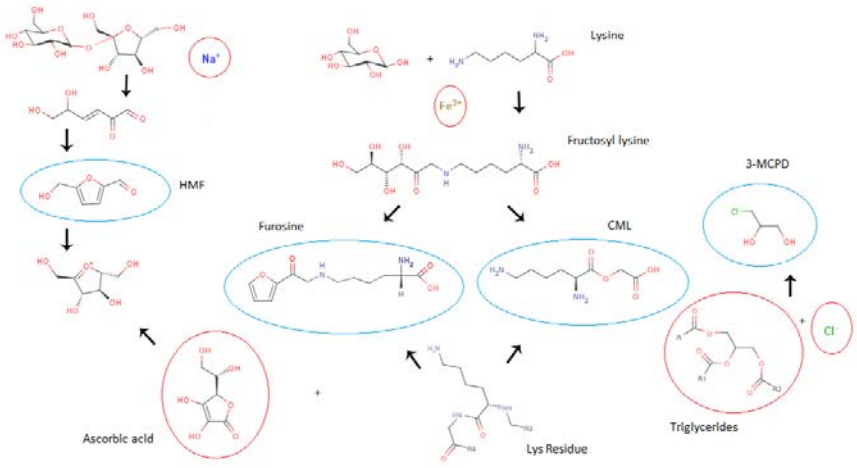

Figure 3 Potential targets for the encapsulation strategy to prevent the formation of harmful MR products.

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1.9.1 Sodium Chloride

The effect of salt has been extensively investigated during the last decades. Gokmen and Senyuva (2007) tested the effect of several monovalent, divalent, and trivalent cations on MRPs formation. Generally monovalent and bivalent cations favour a reduction of acrylamide formation, promoting the decomposition of sugars. As a consequence the presence of cations shifts the reaction pathway that proceeded mainly toward the dehydration and pyrolysis of glucose leading to HMF or furans formation. Sodium ions determined the formation of fructofuranosyl cation which is one of most relevant precursor of HMF (Gokmen and Senyuva, 2007; Locas and Yaylayan, 2008). This effect have been widely demonstrated both in commercial dextrose preparations, in cookie model systems in the presence different concentration of NaCl, in partial dehydratated cherry tomato and in grapefruit, respectively (Muratore, *et al.,* 2006; Quarta and Anese, 2010; Xu, *et al.,* 2003).

Sodium chloride influences the formation of 3-monochloropropane-1,2-diol (3-MCPD) in several food matrix reacting with triglycerides, even if the formation of these molecules is usually associated to the formation of thermal induced toxicants and not directly to the MR. The esters of 3-MCPD belong to a group of well known process-induced contaminants derived from mono- and di-chlorinated glycerols (Crews, *et al.*, 2003; Rahn and Yaylayan, 2011). It was previously verified that the ability of sodium chloride to chlorinate glycerol is greatly enhanced in the presence of amino acids and phosphate-containing compounds such as deoxyguanosine monophosphate (Hamlet, *et al.*, 2004a). In addition, amino acid hydrochloride salts have greater ability to chlorinate glycerol than a mixture of sodium chloride and amino acids (Rahn and Yaylayan, 2011). Even if the mechanisms of action are still unclear, lipids react with chloride ions via nucleophilic attack on the less hindered site on glycerol backbone and the leaving group played a fundamental role in the efficiency of the reaction (Rahn and Yaylayan, 2011)

The encapsulation of sodium chloride is potentially suitable for many others food products and for the control of other reaction such as the nucleophilic attack of chloride ion for the formation of 3-MCPD. In canned fish and brine controlling the activity of NaCl through appropriate protein, polysaccharide or lipid -based coating could be a potential smart approach to reduce the formation of this undesired molecule.

1.9.2 Iron and metal ions

Iron salts are frequently added to many food preparations for nutrition purposes. Iron is used as functional ingredient in baby and growth formulas in particular to prevent anaemia and to rise up the poor bioavailability of the iron naturally present in milk (Leclere, *et al.,* 2002). Besides these

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relevant biological properties, ferric ion could be also a key factor in the development of MRPs. Even if the activity of transition metal towards MR is still matter of debate, some aspects are clear. The rate of browning is influenced by metal ions that promote oxidation reactions leading to dicarbonyl compounds (Hayase, *et al.,* 1996) or to complexes able to catalyze browning (Borrelli, *et al.,* 2002; Obrien and Morrissey, 1989). However, in some cases, metal ions are able to suppress browning (Yaylayan and Huyghuesdespointes, 1994) or can be effectively used to catalyze the precipitation and the successive removal of the brown material (Liang, *et al.,* 2009). Ames and Fallico (1999) verified the effect of iron and hexanal in a Phe-glucose model system demonstrating that ferric chloride is particularly active at pH 5 promoting the formation of 3-deoxyglucosone. Kwak and Lim (2004) investigated how a soybean paste model system incorporating a sugar, amino acid, metal ion, and NaCl would affect browning with the pH value controlled to 6.5. They confirmed the results previously obtained by Morita and Kashimura (1991) demonstrating the oxidative effect of transition metals ions in browning enhancement. Ramonaityte, *et al.* (2009) evaluated the activity of transition metal in a lactose-glycine model system at different stages of Maillard Reaction and in particular the distribution of metal both in dialyzable and nondialyzable fractions of the system verifying that Fe, Cu and Zn ions have a clear impact on coloured compounds formation.

In this framework, iron encapsulation may have a dual purpose: on one hand it can prevent ferric ion oxidation; one the other it can hamper its participation to the development of MR. To maximize the efficacy of the encapsulation strategy the coating should melt at the acidic pH of the stomach thus allowing the absorption by the enterocytes of the duodenal lining. To reach the desired goal the coating material and the type of ferric salt must be accurately selected considering the interaction with food matrix and the intensity of thermal process. Choi and co-workers (2009) showed that Fe^{2+} could be trapped inside the inner water droplets and released at a controlled rate in a model system. Hydrophobic capsules, such as waxy material or other lipophilic structures, are useful for the resistance to the thermal load, but they often showed solubility problems in aqueous solutions. Moreover, iron might cause the aggregation and phase separation of charged macromolecules and colloidal particles. Fluidized bed encapsulation and spray drying process can be used to isolate iron (Augustin and Hemar, 2009), while hydrogel made up by whey protein is particular suitable for the delivery of iron salts, where the release of iron is dependent on whether the gel network is filamentous or particulate (Caillard, *et al.,* 2010).

From the food processing point of view, the limitation of iron catalyzing activity is a crucial point not only in the control of the MR, but also to limit lipid peroxidation and ascorbic acid oxidation.

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1.9.3 Ascorbic acid

In milk infant formulas iron supplementation is often associated with ascorbic acid salt for nutritional purposes (Leclere, 2002). The iron-ascorbate system had deleterious action toward protein and amino acids in milk formula promoting their degradation and polymerization (Dittrich, et al., 2006). Ascorbic acid acts as a pro-oxidant in presence of iron or other transition metals that (Khan and Martell, 1967), along with the production of hydrogen peroxide, catalyze the oxidation of ascorbic acid into dehydroascorbate (Almaas, *et al.,* 1997). This reaction implies various modifications in the food, that were first evaluated by Ueda et al. in a food model system (Ueda, *et al.,* 1986), then by Leclere and Birlouez-Aragon (Leclere, *et al.,* 2002) in a whey-lactose model system added or not with iron-ascorbate. These authors showed that iron-ascorbate system not only increased the degradation rate of tryptophan three times, but it also caused the increase of CML formation and lysine blockage. CML has been widely used as marker of protein carboxylation not only in food, but also in human tissue and urine (Ahmed, *et al.,* 1986; Dunn, *et al.,* 1990). Interestingly, Dunn, *et al*. (1990) demonstrated that CML is formed directly by the reaction between ascorbate and lysine residues in model compounds and protein *in vivo.* Metal-catalyzed reaction stimulate the autoxidative degradation of ascorbic acid accelerating the ascorbylation of protein that leads to the formation or MRPs in food and AGE in human tissues confirming previous observation by Thornalley, *et al.,* (1985). Beside the action as a reactant in MR ascorbic acid also acted as precursor of furan upon thermal treatment. In fact ascorbic acid may generate furan from 2-furaldehyde by thermal decomposition (Fan, 2005; Limacher, *et al.*, 2007; Locas and Yaylayan, 2004); in particular the authors stated that it is not recommended to fortify canned and jarred vegetables with vitamin C before thermal treatment (Limacher, *et al.*, 2007).

Ascorbic acid encapsulation can be carried out to prevent its degradation during processing, but also to protect other bioactive molecules from the catalyzing activity of ascorbic acid. According the specific needs, ascorbic acid capsules can be prepared through different methods, such as thermal phase separation, melt dispersion, solvent evaporation and spray drying (Uddin, *et al.,* 2001). Also in this case the different strategies should be evaluated according to the food matrix and to the type of process. Carnauba or candelilla wax capsules are particularly suitable for solid and fat-rich food, while for liquid food at reduced fat content the use of spray-dried capsules with a polysaccharide coating is the most commonly used solution (Bourlieu, *et al.,* 2006; Desobry, Netto, and Labuza, 1998; Mellema, *et al.,* 2006; Murugesan and Orsat, 2012). The loading of ascorbic acid in chitosan nanoparticles prepared by ionic gelation was also reported (Jang and Lee, 2008). There is a huge variety of food where these encapsulated ingredients can be applied however the key

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target for this MR mitigation strategies are the infant formula as ascorbic acid and iron or metal cations are commonly used as functional ingredients in these kinds of products.

1.9.4 Polyunsaturated fatty acids (PUFA)

Many papers dealing with the encapsulation of bioactive lipophilic compounds have been reported in the literature: the main purpose was to prevent their oxidation (particularly for PUFA): to enhance their solubility in water based system, to reduce off flavour formation thus improving sensorial acceptability (McClements, *et al.*, 2009). There are many factors to be considered in the evaluation of encapsulated lipid stability which have been summarized by Waraho *et al.*, (2011b). From MR points of view, preventing the interaction with the aqueous phase, in which catalyzing agents or protein are solubilized, is the most prominent. According to the food composition, the encapsulation of lipids by spray-drying was highlighted by many authors (Faldt and Bergenstahl, 1995; Krishnan, *et al.*, 2005; Serfert, *et al.*, 2012). This process can cause the formation of lipid-protein adducts through various mechanism: radical-radical interactions or through the ability of aldehydes produced by lipid oxidation to interact with amine in reaction such as Schiff base or Michael addition (Rampon, *et al.*, 2001; Waraho, *et al.*, 2011a; Waraho, *et al.*, 2011b).

It is odd to observe that from the macroscopic point of view MR and lipid oxidation had the common fate of browning products formation. The key point of both reactions is the irreversible formation of early stable intermediates such as the Amadori product and the lipid hydroperoxides for MR and lipid oxidation respectively (Zamora and Hidalgo, 2005). Lipid oxidation products influence directly MR, producing several molecules that differ from the ones produced in absence of lipids (Hidalgo and Zamora, 2005). Gokmen *et al.*, (2011) observed that incorporating encapsulated PUFA in bread resulted in a decrease of HMF and acrylamide. They explained these results considering that carbonyls arising from the thermoxidation of PUFA during baking can promote the conversion of asparagine into acrylamide (Zamora and Hidalgo, 2008; Capuano *et al.*, 2010). When the oil is encapsulated, reactive carbonyls are not available for this reaction due to prevention of its thermo-oxidation during baking.

In a meat model-system consisting in various amino acids, sugars and phospholipids, Elmore et al (Elmore, *et al.*, 2002) observed that phospholipids interact with sugars and amino acids, not only with the amino group of polar head of phospholipids, but also thanks to some oxidative breakdown products which are easily formed particularly form long chain polyunsaturated fatty acids such as EPA and DHA. It is well known that the presence of EPA and DHA caused the increase in lipid-derived volatiles, such as aldehydes, alcohols and alkylfurans in many foods (Mottram and Elmore, 2000). The interrelation between lipid oxidation and MR is evident in the

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formation of acrylamide and several papers deal with the mechanism of formation: via acrolein which provide the reactive carbonyl moiety (Gertz, *et al.*, 2003; Yasuhara, *et al.*, 2003); via the reaction of lipid derivatives and asparagines (Zamora and Hidalgo, 2008); and via the influence of lipid oxidation in various fat-rich model systems (Capuano, *et al.*, 2010).

The encapsulation of EPA and DHA represents another milestone not only in the protection of the essential fatty acids from peroxidation but also in the control of MR. This encapsulation process is particularly suitable for milk infant formula, baby food jarred and canned puree and it has been already employed to prevent PUFA oxidation (Heinzelmann and Franke, 1999; Kralovec, *et al.*, 2012). Theoretically, lipid encapsulated with a protein coating had a better solubility and their reaction with amino acid moiety is prevented. Up to now no example of this prevention strategy has been evaluated in details this far.

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2. Objectives

Foods have been heat-treated for many centuries, since our ancestors learned, by trial and error, to master fire for cooking purposes approximately 700 000 years ago, to modify and preserve the organoleptic and nutritional properties of foodstuffs. It is widely accepted by today's anthropology that the invention and continuous development of thermal food treatment had a substantial, if not the major impact on phenotypical, intellectual, societal and economic development of mankind.

All food scientists know very well that food safety and palatability issues require the use of thermal processes for modulation of food raw materials during food processing at industrial and household levels. However, today many consumers considered thermally treated foods as potentially harmful and when in April 2002, Tareke, *et al.,* (2002) described the formation of acrylamide in heated, protein- and carbohydrate-rich foods this idea was further straightened. Different researchers sustain the idea that some thermally treated foods can have negative effect on inflammation and although never proved with certainty, the long term toxicity some compounds is always suspected.

In this framework, food companies need to be pro-active and many efforts were directed towards the reduction of acrylamide concentration in industrial products. As many strategies to reduce the acrylamide presence are possible the mitigation of its presence in different thermally processed foods is still sparking a growing body of interest worldwide and hundreds of papers have been published on the mutter in the last 12 years.

Beside the reduction of the acrylamide itself many of the mitigation measure implied a modification also of the other Maillard Reaction products which are also formed in those products having often significant sensorial implications. The dynamic cross-talking among different pathways of Maillard Reaction and Maillard reaction products formation further complicated the scenario making necessary a more comprehensive approach to understand positive and negative consequences of each action.

The objective of this research was to investigate in a comprehensive and holistic manner the formation of Maillard reaction products and particularly acrylamide, carboxymethyl-lysine (CML) and hydroxymethyl furfural (HMF) in several foods, different model systems using various processing technology.

The food chain approach was widely used, identifying at the different step of the single food production chain some key elements that can influence the formation of these potentially harmful compounds.

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On one hand the effect of formulation with the effect of antioxidants such as curcumin or the use of new ingredients such as soybean okara will be investigated, on the other the potentiality of new mild processing such as radiofrequency heating will be assessed.

Last but not least the use of encapsulation technology by reducing the amount of some key catalyzers of the Maillard reaction will be tested.

Moreover, as the fast and reliable detection of acrylamide still is a bottleneck for routine analysis a fast method using a High Resolution Mass Spectrometry will be also proposed.

The overall aim of the research is to address the current knowledge of compounds that may be formed during the cooking process that may affect health.

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3. Results and Discussion

In this chapter the main findings of each research paper (Appendix A1-A5) are discussed in the general context of providing better and safer thermally treated food products. Feasibility of the different approach and relevance of the various solutions proposed will be discussed considering the Toolbox for acrylamide mitigation whose last 2013 revision was released by the CCIA at the beginning of this year.

3.1 Main findings and implications of the research on the effect of Okara on the formation of Acrylamide and Carboxymethyl-lysine in Bakery Products

Okara is a protein and dietary fibre rich products obtained from soybean after extraction of the water-soluble component. It is considered a healthy ingredient and from the nutritional point of view this is true. However the data of this paper (Palermo, *et al.,* 2012) demonstrated that the formation of MRPs can be enhanced by the use of ingredients containing their precursors. In particular acrylamide and CML are present at significantly higher concentration in soybean products than in the conventional ones in almost all commercial products. Results of this paper confirmed those previously obtained by our group using four rye flours with different extraction rates (Capuano, *et al.,* 2010). Data of this paper clearly indicated that the amount of acrylamide formed during bisquits cooking is related to the amount of precursor (asparagine) present in the rye which is higher in not refined grain. Similar results can be found also comparing wheat and whole wheat flours (Capuano, *et al.*, 2009).

However this evidence about MRPs formation does not imply that the intake of not-refined cereal should be discouraged. It is plenty of evidence that a diet rich of whole cereal is beneficial for human health and all dietary recommendation suggest increasing their consumption. The right trade off can be achieved by appropriate formulation and processing technology. Dietary fibre rich products have a higher water holding capacity (WHC) and it is know that at high water activity the formation of acrylamide and the development of all MR is inhibited. Therefore it is possible for each product to optimize the time/temperature of cooking or using pre-drying steps to obtain safer foods rich in dietary fibre and other bran components with a limited amount of potentially harmful MRPs such as acrylamide.

Finally, as shown by the data in Table 2 a survey on market product in Italy confirmed that soybean containing products marketed for their healthy features, showed a tendency to contain more acrylamide. This was not surprising in consideration of the abundance of precursors (i.e. free amino acids) present in the soybean flour; it is a point of concern related to consumer belief about this type of products.

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Table 2 Amount of acrylamide in commercial samples containing soybean flour compared to the corresponding cereal samples (adapted from Palermo, *et al.*, 2012)

	Soy products [μg Kg^{-1}]	**Control [μg Kg^{-1}]**	Sign diff
Crispbread	198.36±40.50	nd	
Shortbread	341.25±22.57	189.04±7.30	*
Wholemeal biscuits	496.33±7.96	169.58±16.20	*
Toasted bread	48.45±3.66	nd	
Breakfast cereals	64.42±5.93	90.04±5.39	*
Crackers	97.27±4.89	nd	
Dry biscuits	63.16±1.86	272.14±23.81	*
Butter biscuits	743.78±8.06	62.22±7.96	*
Cereal bars	112.04±1.49	85.47±3.71	*
Extruded soy flour	118.77±18.26		

3.2 Main findings and implication of controlling the Maillard Reaction by Reactant Encapsulation: Sodium Chloride in Cookies

This work was developed in the framework of the EU project Prometheus aimed at exploring new solution to limit the formation of potentially harmful Maillard Reaction products in different products (Fiore, *et al.*, 2012). As mentioned in the Introduction session encapsulation can be a useful approach as it is able to hide some of the key reactants delaying their participation to the reaction.

In this paper we demonstrated in a cookies model system that the encapsulation of NaCl by three different hydrophobic coatings prevented the pyrolysis of glucose and the dehydration of fructofuranosyl cation. Data showed that the key point establishing the efficacy of the encapsulation was the thermal resistance of the wall material: the higher the melting point the more pronounced the reduction of HMF formation. Interestingly, this procedure does not modify the cookies sensorial properties: in fact, modulating the type of coating it is possible to block sodium chloride during the reaction time releasing it close to the end of the cooking process.

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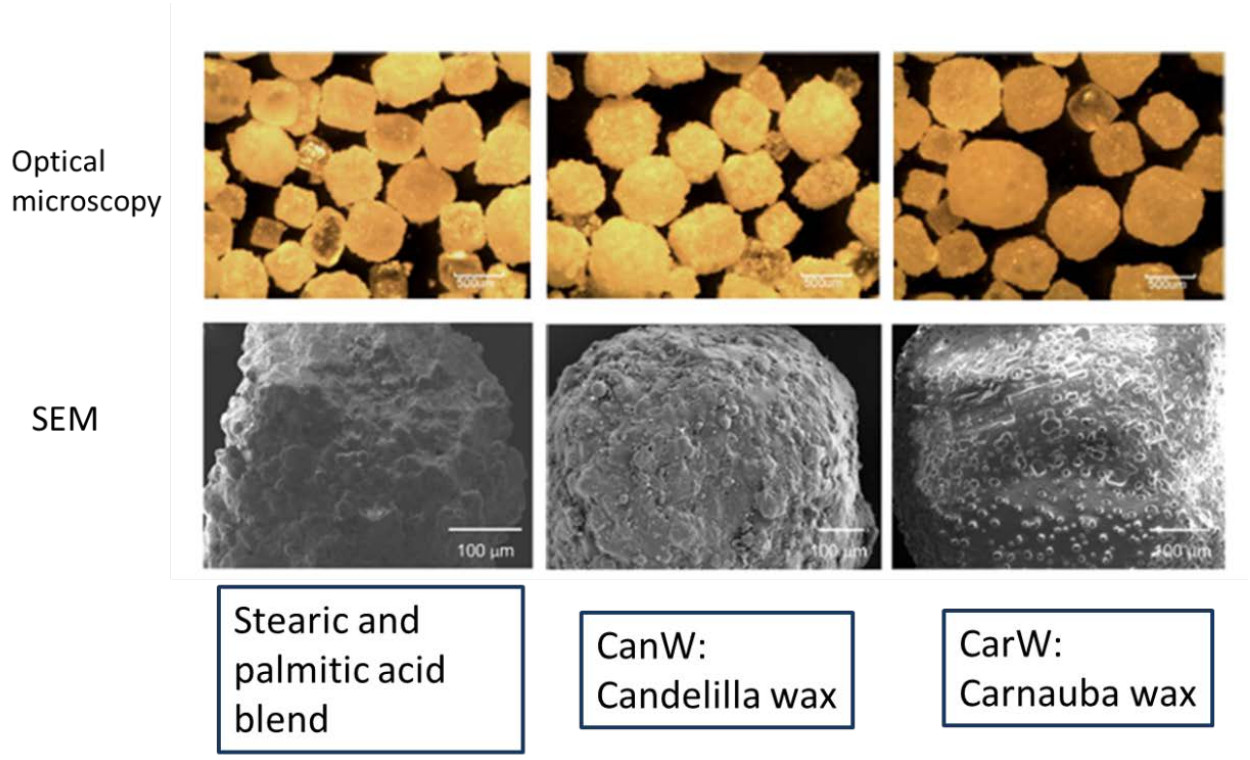

Figure 4 Sodium chloride encapsulated with three different coatings

As shown in Figure 4 the sodium chloride crystals are totally covered by the fatty layer preventing them from the interaction with sugar and therefore inhibiting its catalytic action in the formation of HMF.

The effectiveness of the approach in biscuits depends on the melting temperature of the coating being more effective when the coating melts at higher temperature. Interestingly as sodium chloride is totally released at the end of biscuits cooking time, no sensorial differences are detectable.

In Table 3 some of the possible ways encapsulation can contribute to modulate the MR development were illustrated and in this paper the experiments with sodium chloride fully confirmed the hypothesis.

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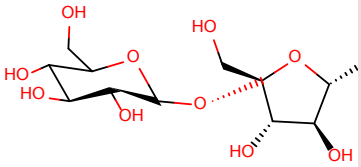
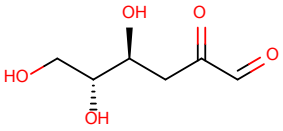
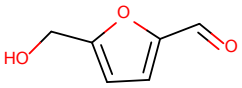
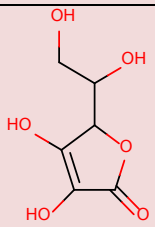
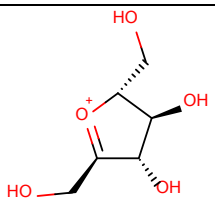
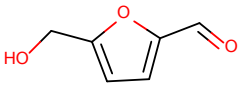
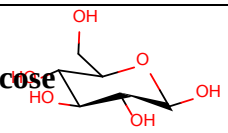
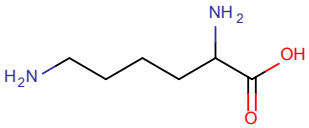
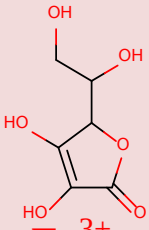
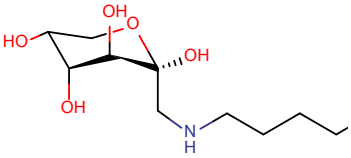
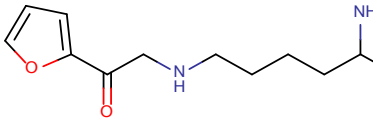
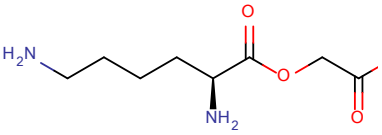
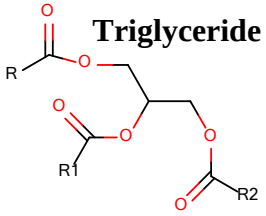
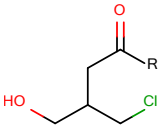
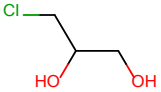
Table 3 Approaches for encapsulation to prevent Maillard reaction products formation

Precursors	Encapsulated Reactants	Intermediates	End Products
 **Sucrose**	Na^+	 3-Deoxyglucosone	 HMF
Ascorbic acid		 Fructofuranosyl cation	 HMF
Glucose   **Lysine**	 Fe^{3+}	 Fructosyl-lysine	 Furosine  CML
 **Triglyceride**	Cl^-	 3-MCPD monoester	 3-MCPD

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3.3 Main findings and implications about the Role of curcumin in the conversion of asparagine into acrylamide during heating

This paper tackled the entangled issue of the role of antioxidant compounds on MR development **(**Hamzalıoğlu, *et al.,* 2013**).** There is a general tendency to think that the presence of antioxidants, which are in most of the case reducing agents, can inhibit the reaction, being some the MR path ways oxidative reaction. However there are a number of limitations and factors that could modify this assumption. First of all the nature of antioxidant compounds as many of them can actively participate the reaction, then the antioxidant concentration as it is known that at high concentration antioxidant compound become prooxidant; third the target MR products that is considered while heterocyclic amines (HA) are generally inhibithed by the presence of antioxidants, HMF is not and many different results have been obtained with acrylamide.

In this paper the case of curcumin was investigated as this compound is very well known for its healthy properties and it is often added to various types of bakery and dairy products. However from the chemical point of view curcumin has a carbonyl function which is potentially able to catalyze the conversion of asparagine into acrylamide.

Data of the paper nicely confirmed the hypothesis: not only curcumin can form acrylamide when added to an asparagine solution, but its effect is additive to that of fructose (Figure 5) suggesting a probable enhancing effect in curcumin–containing bakery preparation.

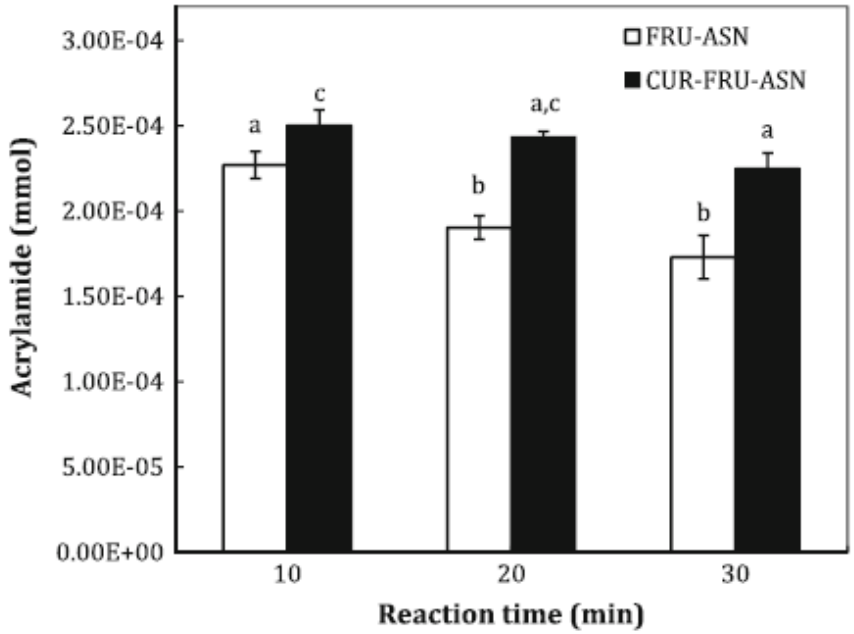

Figure 5 Formation of acrylamide in ternary systems of fructose asparagine and curcumin

In the paper the possibility to detect intermediates of the reaction between curcumin and acrylamide by High-resolution mass spectrometry was also shown for the first time, introducing

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thepossibility of using this new mass spectrometry tool in the investigation of Maillard Reaction products.

3.4 Main findings and implication of the research on the use of radiofrequency oven to mitigate acrylamide formation

There is a mounting interest to develop new technology for industrial and domestic food processing. Reduction of energy consumption and convenience of use are the main driver of device development, however to enter in the market these new technology must prove their efficacy as far as the culinary performance and the safety. In this paper (Fiore, *et al.*, 2013) the possibility to use radiofrequency (RF) for domestic food processing was investigated with the aim to verify if using this technology it is possible to reduce the formation of potentially harmful Maillard Reaction product such as acrylamide and prevent the degradation of some vitamins.

The heat transfer using radiofrequency is similar to that achievable with microwave frequency, but while in the latter case the energy is transferred to the water present in the food, the RF device used in this work allowed a selective heating of different food component. For this reason this “intelligent” device could potentially avoid overheating of some parts of the foods which can lead to spoilage of vitamins or formation of acrylamide.

In Figure 6 an example regarding oven potatoes is shown. The two cooking processes where designed to have starch fully gelatinized at the centre of potato cubes (so the same cooking level).

Figure 6 Potatoes cooked by conventional and radiofrequency oven

Thanks to the efficiency of the RF heating this was achieved more rapidly in the RF oven and consequently the surface of the cubes was exposed to a lower thermal treatment and it is less brown.

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As expected the measure of acrylamide concentration confirmed this finding: the acrylamide formed on the surface of potato cubes in the RF oven using a power of 2350 KJ is less than the half of those measured in the conventionally cooked potatoes

This paper paved the way to the marketing of RF oven in the domestic cooking as a device able to favour healthy cooking behaviour.

3.5 Main findings and implication of the methods developed to quantify acrylamide using Orbitrap High-Resolution Mass Spectrometry

Since the discovery of the acrylamide presence in foods in 2002 there was a continuous fine tuning and optimization to develop low cost robust and reliable methodology. The analytical determination of AA in food products is most frequently performed by high performance liquid chromatographic (HPLC) or gas chromatographic (GC) separation methods with mass spectrometric detection (MS), either in selected ion monitoring (SIM) mode or by tandem mass spectrometry (MS/MS) in multiple reaction mode (MRM) using isotope labelled standards.

Looking at the wide literature available on the topic and at our own laboratory experience it is clear that the main bottleneck for routinely acrylamide analysis is not the detection system but the extraction procedure. Particularly in some matrix such as coffee or cocoa based product the recovery is low and highly variable and in all case the addition of internal standard to each samples required to correct for the recovery each measure.

In the present study the High Resolution Mass Spectrometry (HRMS) couple with an Orbitrap apparatus was used with the aim to take advantage by the enormous discrimination capacity of this mass spectrometry detection to avoid any extraction and purification step of the sample. Being the instrument able to discriminate up to the fifth digit after the coma, most of the potential interference is eliminated and it is possible to analyse directly raw watery extracts from the different matrices (Troise *et al.*, 2014)

Moreover the procedure is so stable and robust that also avoid the addition of internal standard results remain satisfactory at least for screening purposes.

Data reported in Table 4 shown how in the four main food categories contributing to acrylamide intake, the data obtained with and without internal standard are comparable and in line with literature data.

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Table 4 Acrylamide concentration measured by HRMS with and without labelled standard

Food	Acrylamide concentration Without labelled standard	Acrylamide concentration With labelled standard	Acrylamide concentration literature data
Biscuits	276 - 313 ng*g^{-1}	262 – 340 ng*g^{-1}	278-313 ng*g^{-1}
			451-510
French fries	270 – 424 ng*g^{-1}	261 – 457 ng*g^{-1}	253-2688
			150-1200
			50-1800
Brewed coffee	12 ± 0.1 ng*mL^{-1}	15 ± 0.2 ng* mL^{-1}	6-16 ng* mL^{-1}
			5-12
			14-21
Ground coffee	140 ± 3.6 ng*g^{-1}	159 ± 8.1 ng*g^{-1}	45 – 374 ng*g^{-1}

3.6 Physiological relevance of dietary Maillard reaction products

The studies on the biological effects of compounds specifically formed during processing were traditionally focused on the potential harmful effects as they are considered somehow artificial therefore potentially toxic.

However the evidence of the beneficial effects exerted by some processed foods, such as coffee, promoted the studies aimed at investigating the mechanisms and the compounds responsible for the beneficial effects. Some small molecular weight compounds such as pronyl-lysine and *N*-methylpiridinium, as well as some quinide compounds derived from chlorogenic acids have been identified. (Van Boekel *et al.*, 2010)

Recently the structures of two classes of food melanoidins such as those present in coffee (Gniechwitz, *et al.*, 2007; Reichardt, *et al.*, 2009; Bekedam, *et al.*, 2007 and 2008) and in bread crust (Somoza, 2005; Borrelli, *et al.*, 2003) were elucidated allowing to shed some light on the possible biological activities of these compounds.

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3.6.1 Effect of dietary Maillard Reaction products on liver functioning

The effect of dietary MRPs on the functioning of the liver detoxification system is of particular relevance as literature suggests considerable evidence for an association between impaired detoxification and the risk for various diseases, such as some types of cancer (Fontana, *et al.*, 2009, Lacko, *et al.*, 2009; Pongtheerat, *et al.*, 2009), Parkinson's disease (Golbe, *et al.*, 2007) or chronic immune dysfunction syndrome (Wolkenstein, *et al.*, 2005).

Heat treated-foods and even MRP structures formed therein have been demonstrated to modulate Phase I and Phase II enzymes in animal feeding trials. Kitts and colleagues (1983) first reported decreased Phase I aryl hydrocarbon hydroxylase activity in small intestinal enterocytes of mice which were fed an experimental diet containing 2% MRPs for 10 weeks. MRPs were prepared by heating an equimolar mixture of glucose and L-lysine at 121 °C for four hours. Pintauro and Lucchina (1987) administered a heat treated protein, egg albumin, to rats for 10 weeks and also demonstrated a decreased activity of the Phase I enzyme aminopyrine *N*-demethylase in small intestinal enterocytes, whereas hepatic Phase I benzo[alpha]pyrene hydroxylase activity was significantly increased. Although both feeding trials from Kitts, *et al.*, (1983) and Pintauro and Luccina (1987) clearly indicated an effect of MRPs on detoxifying enzymes, the active principles were still unknown. Wenzel, *et al.*, (2002) administered heat treated casein that was selectively fortified with the MRP N^{ε}-carboxymethyllysine (CML). After a feeding period of 10 days, Phase II glutathione *S*-transferase (GST) activity in rat colonic enterocytes and kidneys increased by 64% and 86%, respectively, compared to control animals on a standard diet containing equivalent amounts of non heated casein. However, the heat-treated casein did not only contain CML and other MRPs might have contributed to this Phase II enhancing effect. When CML as a purified, non-protein linked compound was tested for its GST modulating activity in Caco-2 cells, a statistically significant decrease of 10% was observed after the cell's treatment with 0.5 µM CML for 48 h (Faist, *et al.*, 2002). Thus, CML is unlikely to be a potent inducer of Phase II GST activity. In order to clarify whether the Phase I / II modulating activity is more affected by high or by low molecular weight compounds, Faist, *et al.*, (2002) tested various melanoidins fractions of different molecular weight prepared from hot water extracts of roasted caraffa malt in Caco-2 cell cultures. The low molecular weight fraction of <10 000 Da was most effective in activating Phase I NADPH-cytochrome c reductase and Phase II GST activity (+122% and +33% vs control, respectively). The majority of the mid molecular weight compounds tested showed an activating effect on Phase I NADPH-cytochrome c reductase and an inhibitory effect on GST activity. These effects were most pronounced for compounds of up to 70 000 Da and >200 000 Da, but less distinct for fractions of an average molecular weight of 100 000 Da.

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Lindenmeier, *et al.*, (2002) first identified an MRP structure with strong Phase I and Phase II modulating activities that was formed in bread crust upon heat treatment. In accordance with the well accepted fact of antioxidants being potent inducers of Phase II enzymes through cellular binding to the "antioxidant responsive element" (ARE), this compound was also characterised as the key antioxidant in bread crust. Briefly, application of an *in situ* antioxidant assay to solvent fractions isolated from bread crust revealed the highest antioxidative potential for dark brown, ethanol soluble compounds. Both bread crust and, in particular, the intensely brown, ethanolic crust fraction induced a significantly elevated Phase II GST activity and a decreased Phase I NADPH-cytochrome c reductase activity. Antioxidant screening of Maillard-type model mixtures, followed by structure determination revealed a pyrrolinone reductonyl-lysine, abbreviated as pronyl-lysine, of which high concentrations were quantified in the bread crust (62.2 mg/kg), whereas low concentrations were analysed in the crumb (8.0 mg/kg). Exposing Caco-2 cells for 48 h to either synthetically pronylated albumin or to purified pronyl-glycine significantly increased Phase II GST activity by 12% or 34%, respectively, thus demonstrating for the first time that "pronylated" proteins as part of bread crust melanoidins act as monofunctional inducers of GST, serving as a functional parameter of an antioxidant, chemopreventive activity *in vitro*.

Next to bread crust and malt, coffee brews were also studied for their effects on Phase II enzymes (Somoza, *et al.*, 2003). In this study, *N*-methylpyridinium iodide was identified as the key compound modulating Phase II GST. *In vivo* effects of a decaffeinated coffee beverage and *N*-methylpyridinium iodide were tested in a 15-day animal trial on rats. As a result, feeding of 4.5% coffee beverage resulted in an increase of Phase II GST and UDP-glucuronyl-transferase activity by 24% and 40%, respectively, compared to animals fed the control diet. Animals on the N-methylpyridinium diet showed an increase in liver Phase II UDP-glucuronyl-transferase of 65% compared to controls. The mechanism by which *N*-methylpyridinium ions induce Phase II enzymes was recently identified by Böttler, *et al.*, (2009) who showed that this compound effectively induced the gene transcription and translocation of Nrf2, a major transcription factor leading to the expression of antioxidant and Phase II enzymes, such as GST. These results are in line with findings reported by Cavin, *et al.*, (2008) who demonstrated an induction of Nrf2-mediated cellular defence and alteration of detoxifying enzyme activities as mechanisms of chemoprotective effects of coffee in the liver of rats. However, controlled intervention trials are still needed to verify the contribution of *N*-methylpyridium ions and potential other health beneficial compounds in coffee to the reduced risk for diseases such as various types of cancer which are associated with an endogenous load of reactive oxygen species and decreased activities of detoxification enzymes.

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3.6.2 Anticarcinogenic activity

The potential anticarcinogenic activity of food compounds is usually assessed by inhibitory activity on the growth of human tumour cells, mutagenicity tests as well as by investigating the effect on DNA oxidation and on the mutagen-activated protein (MAP) kinase cascade. Traditionally, compounds formed upon thermal treatment were mainly considered for their potential carcinogenic activity (i.e. acrylamide or heterocyclic amines). However there are some *in vitro* studies performed with the chemically characterized Maillard-type chromophores formed under mild heating conditions. These compounds were shown to be potent inhibitors of the growth of human tumor cells in the low micromolar range, causing tumour cell cycle arrest and apoptosis induction. This effect is due to their ability to suppress the induction of the MAP kinase cascade, one of the major signalling pathways in the regulation of cell growth (Marko, 2007). Antimutagenic properties of MRP or melanoidin mixtures have been noted by Kim, *et al.*, (1986) and were attributed to the inhibition of mutagen absorption or to the inhibition of mutagen activation (Powrie, *et al.*, 1986).
Oxidative stress and subsequent DNA damage can be considered as potential biomarkers to investigate the mechanisms of the potential anticancer activity of whole foods.

3.6.3 Prebiotic activity

The potential action of melanoidins as prebiotic deserves more in depth studies. Prebiotics are non-digestible food ingredients that stimulate the growth or activity of bacteria in the digestive system, which are beneficial to the health of the body. Usually this term refers to soluble dietary fibre (fructo-oligosaccharides and inulin) that is able to increase the concentration of lactobacillus and bifidobacteria. Actually also food melanoidins, as part of food indigestible material that reach the lower gut and can be metabolized by the gut microflora, should be considered as potential prebiotic material. This concept was first investigated by Ames and coworkers (1999). They used glycated BSA as a test substance and no prebiotic activity was found. Borrelli and coworkers (2004) demonstrated that the high molecular weight fraction enzymatically extracted from bread crust increased the growth of Bifidobacteria and a similar effect could be observed using the coffee silverskin, a by-product of coffee roasting very rich in coffee melanoidins (Borrelli, *et al.*, 2004).

Recently a series of papers published by the group of Bunzel (Gniechwitz, *et al.*, 2007a, 2007b, 2008; Reichardt, *et al.*, 2009) showed that the high molecular weight fraction from coffee brew contains three fractions that can be distinguished into galactomannans, arabinogalactans and melanoidins. The chemical characteristics of these fractions depended on roasting conditions, but also on the coffee preparation procedure. They demonstrated that coffee melanoidins behave as a soluble dietary fibre since they are fermented by the gut microflora. High amounts of acetate and

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propionate were produced after microbial degradation of high molecular weight components from coffee.

It can be hypothesized that polysaccharide-rich melanoidins such as those present in coffee are preferentially metabolised by Bifidobacteria while protein-rich melanoidins, as obtained by protein glucose mixtures or milk-like systems, are good substrates for protein metabolising bacteria predominantly present in the descending tract of the colon.

4. Conclusions and future perspectives

The results published in the research papers collected in the Appendix of this thesis contributed to elucidate some key aspects of acrylamide, HMF and CML formation in different foods.

Although each of the paper reported in this thesis dealt with very specific aspects in different foods the overall take home message is that the development of Maillard reaction could be modulated according the specific requirement of the specific food of interest.

To this purposes several formulation strategies and advanced techniques such as encapsulation and radiofrequency heating can be adopted. Mild technologies have a long history as possible tool to inhibit MR development, while encapsulation as strategy to control the reactivity during heating or shelf life, represent a totally new concept.

4.1 Mitigation strategies effectiveness in the different foods

Considering the ubiquitary presence of acrylamide in many pillar components of our diet it is not possible to eliminate it from our diet. Nevertheless, as its toxicity is out of discussion it is advisable to work aiming at its reduction at the lowest possible concentration. A major part of acrylamide is produced during home processing of food; therefore is not under authority control and the issue can be only tackled by educational campaign such as the one showed in the following Figure 7 and 8 recommending for example to fry at golden yellow colour and/or avoiding to over-toast the bread slices at breakfast.

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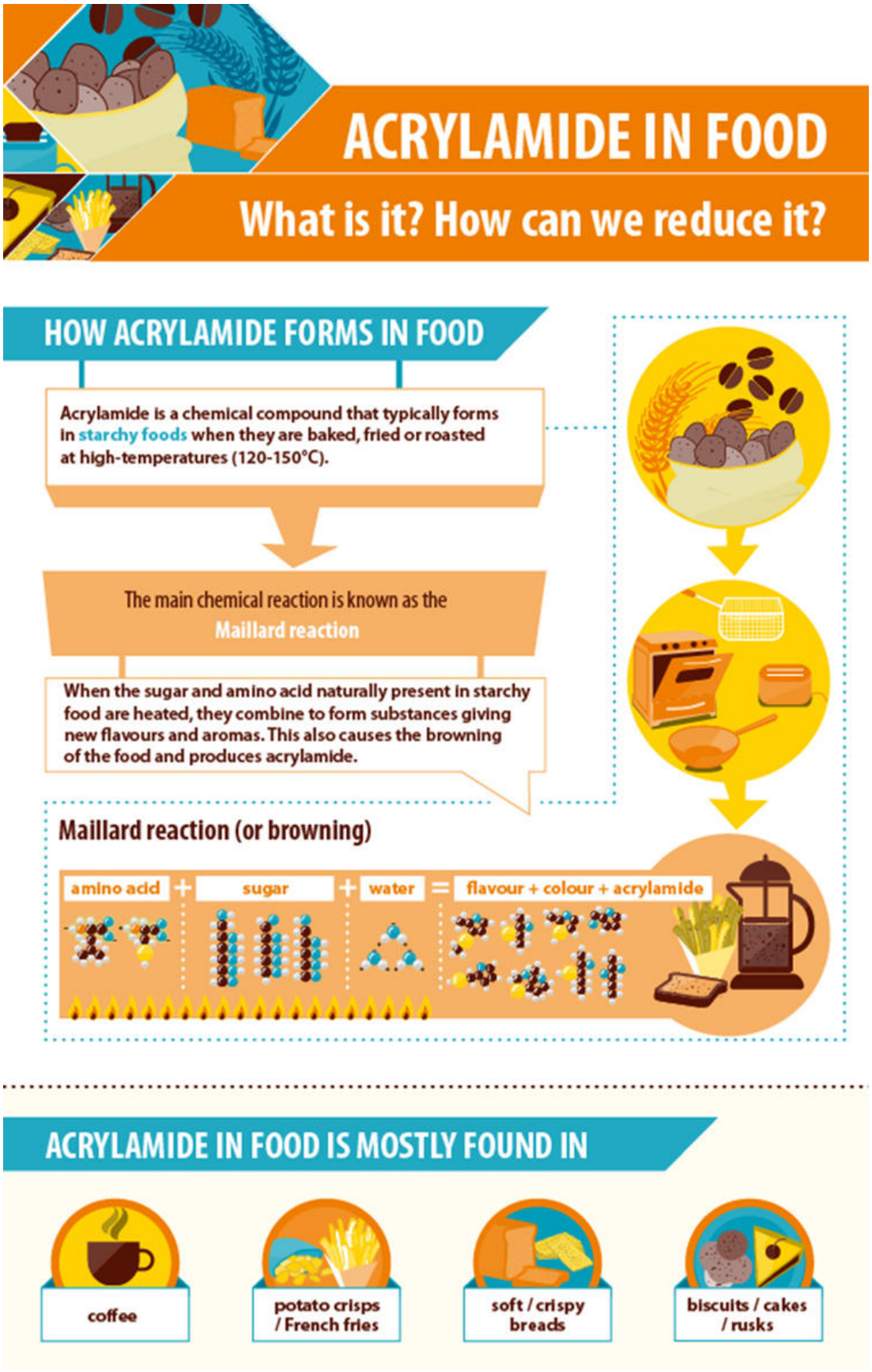

Figure 7 Example of educational brochure released by EFSA

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Figure 8 Example of educational brochure released by EFSA

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The situation is different for food which is thermally treated at industry that in this case is responsible of selling together with their products also potentially harmful compounds. For this reason the Association of the Food Companies worked a lot with EU commission and Academia to develop instructions and guidelines for the food companies operating in the different sector. The final product of this work is the CIAA Acrylamide Toolbox which is an instrument providing national and local authorities, manufacturers (including small and medium size enterprises, SMEs) and other relevant bodies, with brief descriptions of intervention steps which may prevent and reduce formation of acrylamide in specific manufacturing processes and products.

Figure 9 General frame of the Toolbox

It is in particular intended to assist individual manufacturers, including SMEs with limited R&D resources, to assess and evaluate which of the intervention steps identified so far may be helpful to reduce acrylamide formation in their specific manufacturing processes and products.

In **Figure 9** the general frame of the Toolbox is shown, then for each food product a box highlighting the main achievements and the ongoing studies are discussed.

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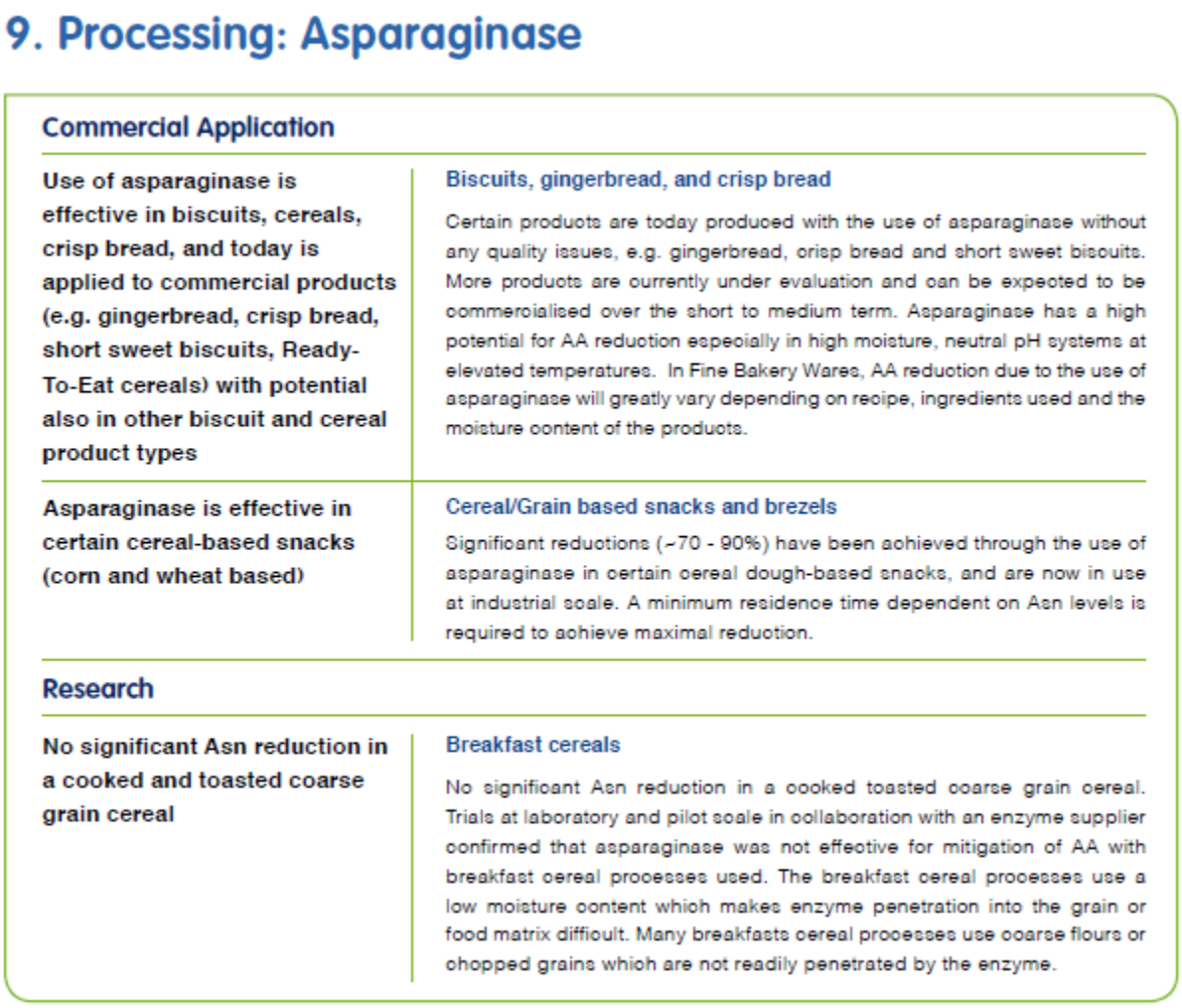
In **Figure 10** the case of Asparaginase during the processing of cereal based product is shown.



9. Processing: Asparaginase

Commercial Application

Use of asparaginase is effective in biscuits, cereals, crisp bread, and today is applied to commercial products (e.g. gingerbread, crisp bread, short sweet biscuits, Ready-To-Eat cereals) with potential also in other biscuit and cereal product types

Biscuits, gingerbread, and crisp bread

Certain products are today produced with the use of asparaginase without any quality issues, e.g. gingerbread, crisp bread and short sweet biscuits. More products are currently under evaluation and can be expected to be commercialised over the short to medium term. Asparaginase has a high potential for AA reduction especially in high moisture, neutral pH systems at elevated temperatures. In Fine Bakery Wares, AA reduction due to the use of asparaginase will greatly vary depending on recipe, ingredients used and the moisture content of the products.

Asparaginase is effective in certain cereal-based snacks (corn and wheat based)

Cereal/Grain based snacks and brezels

Significant reductions (~70 - 90%) have been achieved through the use of asparaginase in certain cereal dough-based snacks, and are now in use at industrial scale. A minimum residence time dependent on Asn levels is required to achieve maximal reduction.

Research

No significant Asn reduction in a cooked and toasted coarse grain cereal

Breakfast cereals

No significant Asn reduction in a cooked toasted coarse grain cereal. Trials at laboratory and pilot scale in collaboration with an enzyme supplier confirmed that asparaginase was not effective for mitigation of AA with breakfast cereal processes used. The breakfast cereal processes use a low moisture content which makes enzyme penetration into the grain or food matrix difficult. Many breakfasts cereal processes use coarse flours or chopped grains which are not readily penetrated by the enzyme.

Figure 10 Use of Asparaginase to mitigate acrylamide formation in cereal based product

Until the end of 2013 there was within the scientific community and the food experts the feeling that a maximum limit for some products (potato crisp and some type of bread) would be set by EFSA, however after the release few weeks ago of the results of the public consultations performed by the CONTAM Panel this possibility seems to be more far.

In fact, the Panel provided several recommendations such as:

- to improve the reporting the regarding the mode of preparation of the products before acrylamide analysis;
- to perform diet studies in order to improve exposure assessment, since they provide a more accurate indication of AA levels in food as prepared and consumed at home;
- to collect data on urinary metabolites levels from individuals participating in the duplicate diet studies for the purpose of validation of the biomarkers;

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- to run up to date epidemiological studies to confirm or refute the inverse relation between dietary acrylamide intake and birth weight and other markers of foetal growth observed in two studies.

On the other hand the Panel concluded that the current levels of dietary exposure to AA are not of concern with respect to non-neoplastic effects. However, although the human studies have not demonstrated AA to be a human carcinogen, the margins of exposure (MOEs) across dietary surveys and age groups indicate a concern with respect to neoplastic effects.

4.2 Regulatory perspectives about dietary Maillard reaction products

Consumer perception of (industrial) processing is rather negative, probably due to the large attention to formation of undesired compounds, but clearly processing also leads to the formation of compounds with beneficial properties. Moreover, industrial processing can be controlled and optimized much better through the application of kinetic principles than household cooking. Epidemiological data indicative of positive effects for consumers are merely available on whole foods but information on if and how these foods are processed is usually not available. However, there is a lack of information about the food processing in epidemiological studies and epidemiological studies should be designed for addressing this purpose as also highlighted by the last public consultation performed by the EFSA CONTAM Panel.

Further work is required to find or synthesize pure standard compounds to enable the conduct of more accurate mechanistic studies and to further identify other bioactive or functional compounds, thus providing stronger evidence of the beneficial effects of food processing.

It appears that many individual compounds as well as compounds from model reactions or whole foods have been analysed in vitro for health beneficial characteristics. The major problem is that it is as yet not clear if these effects can be directly interpreted as health beneficial for the consumer, because metabolism by the gastrointestinal microflora, bioavailability or degradation by human metabolism are of great influence.

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4.3 Future perspectives

A clear understanding of the mechanisms underlying the biological effect is essential for obtaining the intended beneficial effect in the food product. If such knowledge becomes available, new technologies may provide new opportunities to deliver health, quality and safety in food systems.

The translation of consumer perceptions (particularly flavour, texture, the presence of health promoting components) into manageable industrial scale technologies is a major challenge for the food industry and it is a limitation of the state of the current science underpinning modern food processing technology. Systematic studies are required to provide a balanced optimization for the thermal processes that are accepted and widely used by the food industry in terms of food safety, providing acceptable risk and the desired benefits that are satisfactory to both to the consumers and food safety risk managers (i.e. nutritional and organoleptic quality, release of bioactive or functional compounds formed during food processing).

5. **Novel Scientific Results**

- It has been demonstrated, in a cookies model system, that the presence in bakery products of potentially healthy ingredients such ad bran fraction, protein-rich by product such as okara, or antioxidant like curcumin can lead to an increase of acrylamide concentration.
- It has been proved, by the example of sodium chloride and HMF, that the use of encapsulation can be a smart strategy to reduce the available reactants and to modulate the concentration of Maillard Reaction Products.
- It has been concluded that radiofrequency heating providing a controlled heat transfer can result in food cooking with less acrylamide amount.
- It was the first time to use the high resolution mass spectrometry for acrylamide quantification. This new method was proved to be a powerful tool allowing minimizing the extraction steps and the use of deuterated internal standard in the investigation of Maillard Reaction products.

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6. Summary

The quality of food, from the nutritional, microbial safety point of view and sensory aspects depend on a range of variables from farm to fork, including the quality of the raw material, processing techniques, packaging and cooking. The main purpose of industrial food processing is to provide safe and high quality food as demanded by the consumer (Moskowitz, *et al.*, 2009; Luning and Marcelis, 2009). The conduction of thermal processing in an appropriate way is the key to obtain safe food and in many cases also with enhanced nutritional functionality respect to the starting raw material (Van Boekel, *et al.*, 2010). Thermal processes are frequently used in food manufacturing to obtain safe products with a prolonged shelf-life and may have a strong impact on the final quality of foods. Baking, toasting, frying, roasting, sterilization etc. result in desired and undesired effects which all stem from the chemical reactions, namely Maillard reaction (MR), caramelisation and to a minor extent, lipid oxidation, occurring while the foods are heated. One of the purposes of thermal processes is to alter the sensory properties of foods, to improve palatability and to extend the range of colours, tastes, aromas and textures in foods produced from similar raw materials.

Beside the beneficial effect thermal treatment also has some negative effects on food healthy properties: the major concern arising from heating processes come from the formation of hazardous compounds, the so-called food-borne toxicants i.e. compounds that are not naturally present in foods, but that may be developed during heating or preservation and that reveal harmful effects such as mutagenic, carcinogenic and citotoxic effects. Well known examples of these food-borne toxicants are heterocyclic ammines, nitrosamines and polycyclic aromatic hydrocarbons. Recently two food-borne toxicants have gained much interest because of their high toxicological potential and their wide occurrence in foods: Acrylamide and hydroxymethylfurfural (HMF).

The objective of this research was to investigate in a comprehensive and holistic manner the formation of Maillard reaction products and particularly acrylamide, carboxymethyl-lysine and hydroxymethyl furfural (HMF) in several foods and model systems.

The food chain approach was widely used, identifying at the different step of the single food production chain some key elements that can influence the formation of these potentially harmful compounds. On one hand the effect of formulation with the effect of antioxidant such as curcumin or the use of new ingredients as okara has been investigated, on the other the potentiality of new mild processing such as radiofrequency heating was assessed. Last but not least the use of encapsulation technology by reducing the amount of some key catalysers of the Maillard reaction will be also tested. Moreover, as the fast and reliable detection of acrylamide still is a bottleneck for routine analysis a fast method using a High Resolution Mass Spectrometry will be also

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proposed. The overall aim of the research is to address the current knowledge of compounds that may be formed during the cooking process that may affect health.

The results of this investigation have been reported in the five scientific articles reported as appendix of this thesis all of them were published in leading journals of the food science sector. They contributed to elucidate the key aspects of acrylamide, HMF and CML formation in different foods.

The highlights of the published paper can be schematized as follows:

- The presence in bakery products of potentially healthy ingredients such as bran fraction, protein-rich by product such as okara, or antioxidant like curcumin can lead to an increase of acrylamide concentration.
- The use on encapsulation can be a smart strategy to reduce the available reactants and to modulate the concentration of Maillard Reaction Products such as illustrated by the example of sodium chloride and HMF.
- Radiofrequency heating providing a controlled heat transfer can result in food cooking with less acrylamide amount.
- The high resolution mass spectrometry proved to be a powerful tool for acrylamide quantification allowing minimizing the extraction steps and the use of deuterated internal standard.

Although each of the paper reported in this thesis dealt with very specific aspects in different food the overall take home message is that the development of Maillard reaction could be modulated according the specific requirement of a certain food.

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APPENDIX

Research papers

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A.1

Okara promoted acrylamide and carboxymethyl-lysine formation in bakery products

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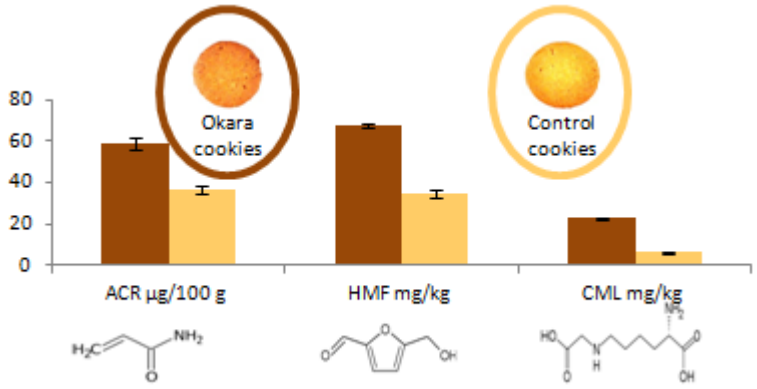

Keywords: Soybean, Okara, Acrylamide, dietary fibre, carboxymethyl lysine

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SUMMARY

Soybean is widely used in bakery product because of technological advantages and recently, soybean-containing products are marketed as functional foods thanks to several health benefits. Okara is a soybean-based ingredient obtained after elimination of the water soluble component from ground soybeans. In this paper the effect of the okara addition to bakery products on the formation of some potentially harmful Maillard reaction products was evaluated.

Cookies obtained by replacing 15% of wheat flour with okara showed a visible browning increase, and a more intense Maillard Reaction development as shown by higher concentration of 5-hydroxymethyl- 2-furaldehyde (HMF) (+100%), acrylamide (+60%) and carboxymethyllysine (CML) (+400%) respect to the control. This phenomenon could be related to the presence in okara of about 50% of insoluble dietary fiber: the fiber reduces water activity during cooking thus promoting Maillard Reaction. To confirm this hypothesis, cookies obtained by replacing 7% of wheat flour with three different types of dietary fiber (cellulose, chitosan and pea fiber) were prepared: these experimental biscuits showed higher Maillard reaction products concentration respect to the control and, in particular, HMF and CML values were directly related to the fiber water holding capacity (WHC).

To extend the observation to the food market a sampling of soybean-containing commercial bakery products was analyzed comparing the concentrations of Maillard Reaction products with those of similar bakery products without soy. Soybean-containing samples showed higher concentration of acrylamide and CML than corresponding controls.

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INTRODUCTION

Soybean flour and soybean protein isolates are widely used in bakery product because of technological advantages such as water binding, dough conditioning, crust coloration and protein supplementation[1]. Recently, many soybean-containing products are marked as functional foods thanks to the several health benefits attributed to soybean, such as hypocholesterolemic activity, lowering risk of coronary heart disease, reducing prostate and breast cancer risk[2].

Vegetarians or vegans are heavy consumers of soy products[3] and they have lower mean body mass index, mean plasma total cholesterol concentration, mortality from ischemic heart disease, risk for some other diseases such as constipation, diverticular disease[4], metabolic syndrome risk factors associated microinflammation than omnivores[5].

Maillard Reaction Products (MRPs) products are formed during the heat treatment of food[6] and a diet rich of heated processed foods (baked, roasted and fried products) implied the intake of several grams of MRPs which are named dietary AGEs[7].

A significant correlation has been found between ingested and circulating AGEs in humans[8] and several reports of evidence suggested that dietary Maillard Reaction Products are implicated in the development of glycation and inflammation associated diseases such as renal failure, diabetes and Alzheimer's disease[9-11]. Interestingly, higher levels of plasma advanced glycation end products (AGEs) were found in vegetarian people than in omnivores and this phenomenon has not yet been well explained[2].

Bakery products are among the pillars of human diet[13] and the Maillard reaction is the main chemical event occurring in this kind of foodstuffs, during cooking[14]. In bakery products the principal markers of Maillard reaction development are 5-hydroxymethyl- 2-furaldehyde (HMF), acrylamide and carboxymethyl-lysine (CML). Bakery products contain soybean derivatives are widely used in particular by vegetarian people to achieve the recommended protein daily intake.

The aim of this study was firstly to evaluate the effects of the addition to bakery products formulation of okara, which is a soybean products obtained after extraction of the water soluble component, on the formation of potentially harmful Maillard reaction products. Biscuits prepared by partially replacing wheat flour with okara were analyzed measuring acrylamide, CML and HMF formation.

As second step, a sampling of commercial soybean containing bakery products was analyzed aiming at verifying if there is a relationship between the soybean addition and the amount of dietary MRPs.

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MATERIALS AND METHODS

Materials

All chemicals of analytical grade were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless mentioned otherwise. 120 13C3-Acrylamide (isotopic purity 99%) was from Cambridge Isotope Labs (Andover, MA, USA). Oasis-HLB cartridges (30 mg, 1mL) were supplied by Waters (Miliford, MA, USA). Soybean seeds, wheat flour, palm oil and sugar were purchased from local market. Cellulose was from Chimpex Industriale Spa (Italy), chitosan from A.C.E.F. Spa (Italy) , cortical pea fiber from I.T.ALI. Srl (Italy).

Nine different types of bakery products (with or without soy addition) were analyzed: crisp bread, breakfast cereals, cracker, toasted bread, cereal bars, shortbread, wholemeal biscuit, cream biscuits, dry biscuits. Partially defatted and extruded soy flour was analyzed, too. All bakery products were purchased from supermarkets in Italy.

Preparation of okara

According Palermo et al.[15], soybean seeds were soaked in water (ratio 1:10 w:v) at room temperature for 24 hours and soaked soybeans were milled with the same amount of water. The obtained paste was boiled for 30 minutes and the material filtered to separate soymilk from residue pulp. Okara was freeze-dried and finely ground. 100 g of dried okara contained 33.60 g of protein, 9.40 g of fat and 48.78 g of dietary fiber.

Preparation of cookies

The model cookies were prepared according to a recipe described in AACC (American Association of Cereal Chemists) Method. Soy effect was evaluated by replacing 15% of the flour content with dried okara, fiber effect was evaluated by replacing 7.5% of the flour content with dietary fiber in order to obtain the same fiber amount of biscuits with okara: the composition of model systems is reported in **Table 1**.

Shortening, sugar, salt and $NaHCO_3$ were mixed 1 min +1 min + 1 min. NH_4HCO_3 dissolved in water was added to mixer for 20 sec + 20 sec + 20 sec. Flour was added as last ingredient and mixed for 10 sec + 10 sec + 10 sec (total 30 sec). Dough was kept 30 minutes at refrigerator (4°C) because palm oil melts very easily at room temperature and leaks from the dough, but before rolling and molding dough was kept at room temperature for 5 min in order to soften it.

Dough was shaped in a small cylinder having a diameter of 3 cm and thickness of 0.3 cm. Each time same amount of dough (~20 g) were put in a laboratory oven set at 200°C for 14 minutes.

Cookies were freeze-dried and finely ground.

Chemical parameters

Moisture was determined by oven-drying at 105 ± 1°C (AOAC, 1995).

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Quantitative determination of reducing sugar was performed by Fehling solution[16].

For water holding capacity (WHC) determination, 1 g of fiber was mixed with 50 ml of distilled water vigorously for 1 min and then centrifuged for 15 min at 10.000g at 20°C. The supernatant was discarded and the tube was kept inverted for 10 min: results were expressed as grams of holding water per gram of fiber[17].

All determinations were performed three times for each sample.

Instrumental measurement of color

The color was measured by the CIE La*b* system using a Minolta CM2600d (KonicaMinolta, Japan). Six measurements representing the cookies were taken from each sample over a measuring area of 8 mm. The instrument was standardized using standard white plates. Browning is inversely related with b* values.

Asparagine determination

Asparagine content was determined in duplicate according to the modified method suitable for use with the commercially available EZ:faast amino acid kit[18]. Freeze-dried and milling okara sample (0.5 g) was extracted by 2 mL of water and sonicated at room temperature for 10 minutes. The mixture was then centrifuged at 4000g and 100 µL of the supernatant was subjected to solid phase extraction and derivatization steps using the EZ: Faast technology and kit. Chromatographic separation was performed using an HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer, Shellton, CT, USA) and a EZ: Faast column (250 mm ×3 mm, particle size 4 µm) (Phenomenex, CA, USA). The eluents were: A water containing 10mM ammonium formiate; B methanol containing 10mM ammonium formiate. The gradient program was as follows, at a constant flow of 0.5 mL/min: 0.00 min 68%B, 13.00 min 83% B, 13.01 min 68% B, 17.00 min 68%B. The LC flow was split and 200µL/min was sent to the mass spectrometry. MS analysis was performed on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with an electrospray ionization working in the positive ion mode.

Acrylamide determination

The samples were prepared for acrylamide analysis using a procedure described elsewhere[19] with some modifications. 2 g of sample was dispersed into 9 ml of water, d3-acrylamide (100 µL of 20 ppm solution) was added and the mixture was homogenized for 10 minutes in a vortex mixer. The co-extracted colloids were precipitated by adding 1 mL of a 0.68 M potassium hexacyanoferrate(II) trihydrate solution (Carrez I) and 1 mL of a 2M zinc sulfate heptahydrate solution (Carrez II). The mixture was centrifuged at 5000 rpm for 10 minutes; the centrifugation was performed at 4°C to separate fat at the top as solid layer. Aqueous extract was transferred into a tube and the solid residue was extracted twice again with 5 mL of water. After centrifugation, the clear supernatants

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were collected and the extract obtained was eluted through a preconditioned Oasis MCX cartridge at a rate of one drop per second. The extraction procedure was repeated twice for each sample.

LC/MS/MS analysis was performed using API 2000 triple quadrupole mass spectometer (Applied Biosystem Sciex), with a Electro Spray Interface, coupled to HPLC binary micropumps (Perkin Elmer, Series 200). Analytical separation was achieved with an Inertsil ODS-3 column (25×0.46cm, 5µm) (GLC-Sciences, Tokyo, Japan) using isocratic elution with a mobile phase of 0.2% formic acid in water at a flow rate of 0.8 mL min^{-1}. The quantification was carried out in MRM (Multiple Reaction Monitoring) mode at m/z ratios of 72.1 and 75.1 for acrylamide and [2,3,3-d3]-acrylamide, respectively. Moreover an m/z 55.1 and 44.0 and a m/z of 58.0 and 44.0 corresponding to specific molecular fragments of acrylamide and [2,3,3-d3]-acrylamide respectively were monitored. The ions were obtained through fragmentation by specific collision energy of a selected ion precursor, applying a voltage of 4.5 kV.

A delay time of 3 minutes was selected to avoid the introduction of co-extracted matrix components into the MS/MS instrument prior acrylamide elution. The needle and cone voltages were set at 3.0 kV and 100 V respectively. Nitrogen was used as nebulizer gas (12.0 L h^{-1}) and the source temperature was set at 350ºC. Acrylamide was quantified using a linear calibration function that was established with standard solutions of acrylamide and [2,3,3-d3]-acrylamide dissolved in Milli-Q water (25 to 1000 µg/L). Acrylamide contents in sample extracts were calculated from the calibration curve and intercept value, taking into account the recovery calculated by means of [2,3,3-d3]-acrylamide curve. Two injections were performed for each extract.

HMF determination

The analysis of HMF was performed as described by García-Villanova et al.[20] with slight modifications. Extraction protocol was the same of acrylamide extraction protocol, but after third centrifugation, samples were filtered (0.45 µm) and analyzed by HPLC (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of acetonitrile in water (5% v/v) at a flow rate of 1 mL/min under isocratic conditions and a Synergy 4 µm Hydro-RP 80A, 25 9 4.6 cm (Phenomenex) column. The UV detector was set at 280 nm and HMF was quantified using the external standard. Two injections were performed for each extract.

CML determination

CML extraction was performed as described Charissou et al.[21] with slight modifications.

10 mg of grinded samples were reduced overnight at room temperature by adding 100 µL of 500 mM $NaBH_4$ in 0.2M borate buffer, pH 9.2. After addition of the internal standard (100 µL of 1 ppm solution) the reduced samples were hydrolyzed in 5 ml of 6M HCl at 110°C for 24 h. 500µL were dried under vacuum (Speed Vac concentrator, Savant, Farmingdale, NY, USA) and further

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dissolved in distilled water (500 µL), filtered (nylon 0.22 µm) and dried again. The extraction procedure was repeated twice for each sample.

LC analysis was performed as described Schettgen et al.[22] with some modifications, with Micro HPLC Series 200 (PerkinElmer, Shellton, CT, USA), equipment including a binary gradient pump, degasser and autosampler. Chromatographic separation was performed on a Tosoh Bioscience TSK Gel Amide - 80 column (250 mm×2 mm, particle size 5 µm).

The solvents used to prepare the mobile phase were 5 mmol L^{-1} aqueous ammonium formiate buffer, these condition was kept for a run of 10 min.

The mobile phase flow rate was constant at 0.2 mL min^{-1}. Mass spectrometric analysis was performed using a Sciex API 2000 tandem mass spectrometer (MS–MS) with an electrospray ionization (ESI) source working in the positive-ion mode. Instrument control, data acquisition, and evaluation were performed with Analyst 1.3.2 software from Perkin–Elmer. The operating conditions were applied ESI needle potential +5000 V; nitrogen was used as nebulizer gas (at a pressure of 12 psi), as turbo heater gas (500 °C) and as collision gas. Collision gas and curtain gas were set at 10 and 8 instrument units, respectively. Two injections were performed for each extract.

Statistical analysis

Differences among model system cookies were determinate by analysis of variance and Duncan's multiple range test ($P \leq 0.05$).

Differences between products with and without soybean addition were determinate by Student's t-test ($P \leq 0.05$).

RESULT AND DISCUSSION

Formation of Maillard Reaction Products in biscuits added with soybean

In the first set of experiment the effects of the okara addition to a biscuits model system formula were evaluated. The okara ingredient used in this study contains about 50% DF and more than 30% proteins. According to previous works[23], the main constituent in okara is dietary fiber but it, also, presents high protein content so it is useful to fortify bakery products particularly those intended for vegetarians and vegans[25].

Okara samples used in this study had an asparagine concentration of 0.12 g kg^{-1} which is a value in line with those usually present in wheat flour[26-27]. On the other hand, okara is richer in reducing sugar than wheat flour: our okara sample has 3.83 g/100g of reducing sugar while the average content of reducing carbohydrates in wheat flour was 1.7 g/100g[28].

In **Table 2** MRPs content and b* colorimetric values in model cookies are shown. During the cookies baking, water of dough was completely removed (final moisture is below 0.5%) so there

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were no differences in term of the final relative humidity and in term of water activity among model systems biscuits. Maillard reaction products concentration data showed that okara-containing cookies had higher browning and significant higher values of HMF (+100%), acrylamide (+60%) and CML (+400%) in respect to the control: the MRPs content was influenced only by the flour replacement and not by the moisture.

Formation of Maillard Reaction Products in biscuits added with dietary fibre

To explain the increased presence of MRPs in the okara-containing cookies, other recipes replacing okara with different types of dietary fiber were prepared.

The okara sample used in this study has 48.8 % of dietary fiber and previous work indicate that okara polysaccharides contain predominantly galactan, arabinan, arabinogalactan, xylogalacturonan, rhamnogalacturonan, xylan, xyloglucan and cellulose[29]: these polysaccharides bind water so, during biscuits cooking, they reduces water availability and for this reason they have the potential to speed up Maillard reaction.

Results of **Table 2** demonstrated that the addition of insoluble dietary fiber to cookies increased the concentration of acrylamide and CML respect to the control. The DF used in the formulas have different WHC: cellulose showed the highest values of WHC namely 8.55 g of water per g of fiber. Cortical pea fiber and chitosan had WHC of 5.66 and 3.05, respectively.

Interestingly, the type of fiber did not significantly modify the acrylamide content, while dietary fibre differently influenced HMF and CML values in respect to okara: in the case of chitosan biscuits the HMF concentrations was below the control; while all dietary fibre increased the CML at much lesser extent in respect to okara.

As showed in **Figure 1** significant correlation was found between the fiber WHC and HMF concentration in cookies (R^2 = 0.9157) and between fiber WHC and CML content in cookies (R^2=0.9735). This figure indicated that the greater the fiber WHC the faster the Maillard Reaction development as the water activity decrease.

Previous works studied the polysaccharides addition in bakery products (bread, in particular) and they reported a higher browning related to the development of Maillard reaction. Anil[30] added 5-10% of hazelnut testa to wheat flour bread and found differences in terms of crust colour. Similar results were also reported by Gómez et al.[31]: they analyzed bread added with several type of dietary fiber. In general, no significant colour differences were observed between the control bread and the 2% fibre-supplemented bread but breads with 5% of fibre produced darker crumb. A good correlation between Maillard product content and browning development has been repeatedly reported[32,33].

The possible reason for the dramatic increased concentration of CML in soybean containing cookies

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could be related to the okara lipid composition. Okara fatty acids profile presents polyunsaturated fatty acids such as linoleic acid (54% of the total fatty acids content) and linolenic acid (9% of the total fatty acids content)[34] and many papers[35-37] demonstrated that the presence of polyunsaturated fats can markedly contribute to the MRPs formation which is higher than in system containing fats less easy to oxidize.

A greater Maillard reaction in presence of soy added products was also reported by Guerra-Hernandez et al.[38] Their analysis of infant cereal foods reported a higher furosine concentration in those containing soybean.

Maillard Reaction Products concentrations in commercial biscuits containing soybean

Table 3 showed acrylamide concentrations determined in a sampling of soybean-containing commercial samples and correlates them with data on acrylamide concentration reported by European Food Safety Agency[39]: EFSA database reported a large number of samples for each type of bakeries, but no products with soybean added.

Data reported in **Table 3** for wheat based product demonstrated that our data are quite in line with the literature being well inside the range reported by EFSA for each product category. Interestingly, looking at the comparison between conventional and soybean-containing products in most of the case those containing soybean showed higher acrylamide content. This is even more intriguing considering that in all the cases but one the water content is higher in soybean products and it is known that the lower the water concentration of the bakery products the higher the formation of acrylamide. On the other hand, the sample denominated “dry biscuits” behave as expected: having lower water content than the soybean corresponding product (2,62±0,09 vs 4,05±0,05) also showed higher acrylamide concentration (272.14±23.81 vs 63.16±1.86). A similar behavior was also observed for breakfast cereal product: lower water content and higher acrylamide concentration in the conventional products.

Very high acrylamide concentration was found in butter biscuits with soy addition: this feature is also related to the fact that in this product sucrose was replaced by rice syrup which is a mixture of reducing sugars (glucose, maltose and maltotriose)[40].

Table 4 shows HMF and CML concentrations and correlates them with proximate composition.

Unlike what observed for acrylamide, in most of the analyzed food, HMF content was higher in products without soy, than in similar products with soy. For example crisp bread, shortbread and butter biscuits samples without soybean showed higher values than samples with soy addition. This could be explained with higher sugar content in analyzed products without soybean as HMF is formed from the degradation of sugars at high temperatures[41] . HMF was not detected in wholemeal

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biscuits and breakfast cereal with soybean addition. Toasted bread with soy addition showed not only higher acrylamide content but also higher HMF content than toasted bread without soybean addition. Literature data for HMF content in bakery products are in line with those here obtained: dry biscuits values range from 0.5 to 182.5 mg/kg[42-44]; breakfast cereal values range from 3.7 to 193 mg/kg [43]; toasted bread values range from 11.8 to 90 mg/kg[43,45].

In almost all analyzed products with soybean, CML content was much higher than in similar products without soy. This is probably due to the presence of unsaturated fats which can be thermo-oxidized during baking and promoted CML formation. One significant exception occurs with toasted bread: although HMF and acrylamide contents were higher in the toasted bread with soybean addition, in toasted bread without soybean addition Maillard reaction was more advanced with an higher CML value. Very few data are reported in the literature about the content of CML in bakery products and they are not always in agreement with our finding. Hull et al.[46] reported similar values in crackers (1.1 mg/kg) but higher values in shortbread and in breakfast cereal (61.8 and 54.2 mg/kg, respectively).

Summarizing, okara is considered a health promoting and technologically interested food ingredient because of its macronutrient composition and for the high presence of insoluble dietary fiber. On the other hand, the data of this paper highlighted that okara addition in bakery products promoted the formation of some MR products. In particular acrylamide and CML are present at significantly higher concentration in soybean products than in the conventional ones in almost all commercial products. This trend is related to the water holding capacity of the okara fibre but it is also related to the protein and lipid moiety of this ingredient.

In this framework, the addition of soy in bakery products could raise some awareness regarding the possible negative effects to human health. It is well established that plasma concentration of advanced MRPs and in particular acrylamide and CML is correlated to their dietary intake[47]. The findings here reported could explain the evidence that people consuming a vegetarian or a vegan diet has higher concentration of plasma AGEs than omnivores[12] as vegetarians are heavy consumers of soybean containing products.

Finally data in this paper contributed to improve the knowledge of MRPs concentration in commercial foods. Nowadays, European Food Safety Agency database reports acrylamide content in a large numbers of foodstuffs, however it does not take into account soybean-based products.

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Table 1. Ingredients of model system cookies containing Okara or different Dietary fibre as detailed in the text

	Control cookies	Soy cookies	Fiber cookies
Wheat flour	80.0 g	68.0 g	74.1 g
Okara		12.0 g	
Dietary fiber (cellulose OR pea fibre OR chitosan			5.9 g
Shortening (Palm oil)	20.0 g	20.0 g	20.0 g
Sucrose	35.0 g	35.0 g	35.0 g
$NaHCO_3$	0.8 g	0.8 g	0.8 g
NaCl	1.0 g	1.0 g	1.0 g
NH_4HCO_3	0.4 g	0.4 g	0.4 g
Water	17.6 mL	19.3 mL	18.4 mL

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Table 2: Acrylamide content, HMF content, CML content, b* value in cookies with okara and with fiber (mean values ± SE). Different letters within the same column indicate significant differences at P<0.05

	Acrylamide (µg/kg dry basis)	HMF (mg/kg dry basis)	CML (mg/kg dry basis)	b*
Control Cookies (wheat flour only)	361.88±19.97 ^{c}	34.81±2.02 ^{b}	6.32± 0.45 ^{d}	27.33±0.47 ^{a}
Okara cookies	588.84±27.54 ^{a}	67.48±1.35 ^{a}	22.84±0.30 ^{a}	24.40±0.42^{b}
Cellulose cookies	513.76±7.75 ^{b}	32.89±3.52 ^{b}	9.98±0.30 ^{b}	27.86±0.31 ^{a}
Pea fiber cookies	540.56±7.84 ^{ab}	28.13±2.80 ^{b}	9.09 ±0.47 ^{b}	24.41±0.52 ^{b}
Chitosan cookies	544.64±8.56 ^{ab}	14.93±0.40 ^{c}	7.66 ±0.14 ^{c}	24.17±0.58 ^{b}

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Table 3: Acrylamide concentrations (µg/kg dry basis) determined in a sampling of commercial soybean-containing commercial samples (mean values±SE) and acrylamide concentration (µg/kg dry basis) reported by European Food Safety Agency (EFSA, 2006). * and ns indicate significant and not significant differences between soybean-containing and not soybean-containing samples at P<0.05.

	Commercial samples			**EFSA reference**
	Soy-containing products	Conventional products (Control)	Sig.	
Crispbread	198.36±40.50	nd		5-2838
Shortbread	341.25±22.57	189.04±7.30	*	5-2949
Wholemeal biscuits	496.33±7.96	169.58±16.20	*	15-499
Toasted bread	48.45±3.66	nd		10-1430
Breakfast cereals	64.42±5.93	90.04±5.39	*	10-440
Crackers	97.27±4.89	nd		5-830
Dry biscuits	63.16±1.86	272.14±23.81	*	10-4256
Butter biscuits	743.78±8.06	62.22±7.96	*	5-3324
Cereal bars	112.04±1.49	85.47±3.71	*	96
Extruded soy flour	118.77±18.26			

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Table 4 MRPs concentration and proximate composition in commercial samples (mean values±SE). * and ns indicate significant and not significant differences between soybean-containing and not soybean-containing samples at P<0.05

	HMF (mg/kg dry basis)			CML (mg/kg dry basis)			Water content (%)			Reducing sugar content (%/dry basis)		
	Soy products	Conventional products (Control)		Soy products	Conventional products (Control)		Soy products	Conventional products (Control)		Soy products	Conventional products (Control)	
Crispbread	4.53±0.28	12.39±0.41	*	18.2±0.19	1.32±0.27	*	2.09±0.19	1.06±0.02	*	2.76±0.01	3.94±0.00	*
Shortbread	3.23±0.31	6.07±1.79	*	1.13±0.26	1.43±0.11	*ns*	2.08±0.09	1.07±0.24	*	21.65±0.02	24.86±0.06	*
Wholemeal biscuits	nd	4.73±0.16		4.14±0.51	1.73±0.60	*	6.13±0.18	2.81±0.04	*	20.24±0.04	15.43±0.01	*
Toasted bread	35.88±0.31	20.43±0.62	*	0.12±0.05	2.75±0.10	*	1.34±0.02	6.63±0.20	*	1.64±0.00	4.71±0.01	*
Breakfast cereals	nd	23.41±0.77		8.68±0.37	1.22±0.14	*	7.20±0.06	2.70±0.05	*	3.34±0.00	16.44±0.01	*
Crackers	1.43±0.02	nd		11.31±0.36	2.96±0.37	*	3.13±0.03	1.91±0.05	*	5.78±0.00	3.06±0.00	*
Dry biscuits	3.61±0.32	3.30±0.17	*ns*	6.22±0.34	0.11 ±0.04	*	4.05±0.05	2.62±0.09	*	18.34±0.01	19.00±0.02	*
Butter biscuits	9.54±0.59	23.74±0.08	*	3.08±0.10	3.94±0.09	*ns*	6.56±0.03	3.08±0.29	*	8.88±0.00	18.37±0.06	*
Cereal bars	2.69±0.06	13.01±0.23	*	4.54±0.16	3.45±0.19	*ns*	12.37±0.28	6.62±0.21	*	26.93±0.09	26.56±0.06	*ns*
Extruded soy flour	4.02±0.45			3.84±0.99			5.57±0.29			11.65±0.04		

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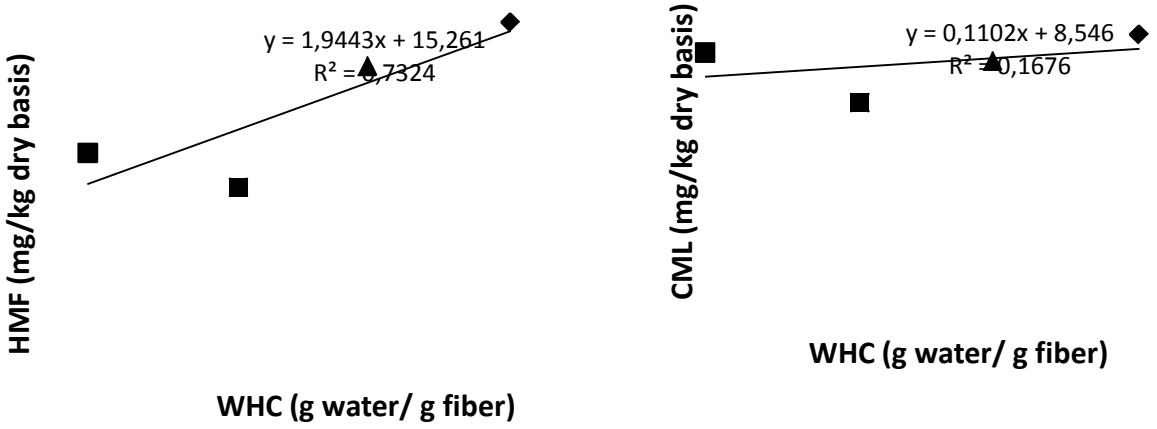

Figure 1. Correlation between fiber WHC and the concentration of Maillard Reaction products. Left panel, HMF; right panel, CML

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A.2

Controlling Maillard Reaction by Reactants Encapsulation: Sodium Chloride in Biscuits

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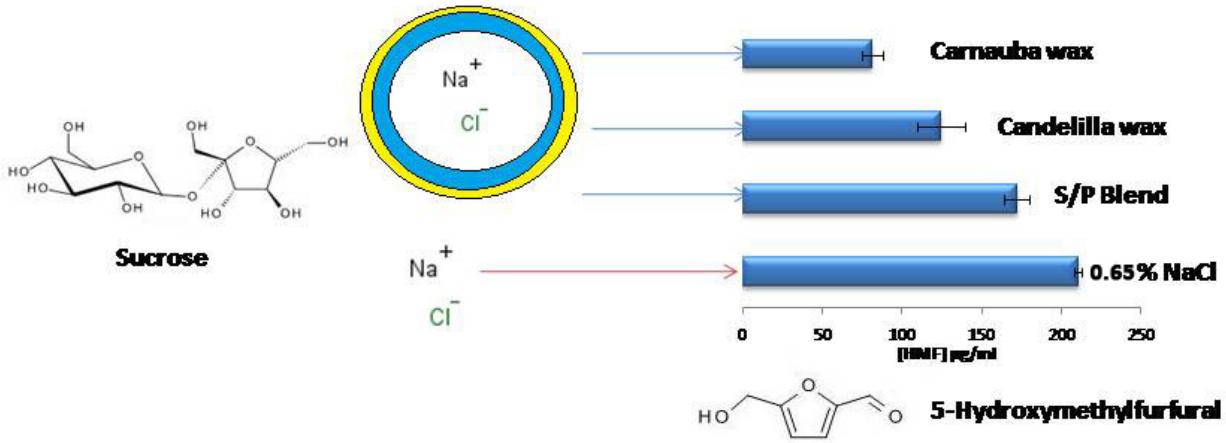

Keywords: Encapsulation, HMF, Maillard Reaction, NaCl

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Abstract

Formation of Maillard Reaction products(MRPs) including 5-hydroxymethylfurfural (HMF) and acrylamide has been an intensive area of research in the last decades. The presence of reactants such as sodium chloride may influence the Maillard Reaction (MR) pathways through the dehydration of various key intermediates. The aim of this work was to test the potential of ingredient encapsulation to mitigate the MR by investigating the case of sodium chloride encapsulation on the HMF formation in biscuits.

Thirteen biscuits were prepared with recipes containing free or encapsulated NaCl. Increasing NaCl concentration from 0% to 0,65%, increases HMF concentration up to 75% , while in presence of encapsulated NaCl the reduction of HMF varied from 18% to 61% due to the inhibition of sucrose pyrolytic decomposition and the fructofuranosyl cation formation. Data demonstrated that the more heat-resistant the lipid-based coating was, the more pronounced the reduction of HMF formation. The results showed that encapsulation represents a useful approach to prevent the formation of potentially harmful compounds in thermally processed foods.

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Introduction

In the last decades the Maillard Reaction (MR), i.e. the reactions between reducing sugars and amino acids, proteins or simple amines have been intensively studied. The Maillard Reaction pathways, which are fundamental in heated food, lead to the formation of many Maillard Reaction Products (MRPs): flavoring compounds, non enzymatic browning products, high molecular weight compounds (melanoidins), reductones, amino-reductones[1]. Some of these compounds gave desired features to the final products, but others are mutagenic, carcinogenic or cytotoxic[2-4].

Acrylamide and 5-hydroxymethylfurfural (HMF)are potentially toxic compounds; in particular acrylamide has been classified by the International Agency for Research on Cancer (IARC) as a probably carcinogen to humans[5,6] ; while there are contradictory findings on the possible carcinogenicity of HMF. HMF in vitro genotoxicity was observed when a specific sulphotransferase catalyzes for the formation of the reactive metabolite 5-sulphoxymethylfurfural[7].

Several mitigation strategies have been proposed to limit the formation of HMF and acrylamide[8]. The mitigation procedures are addressed to change the process conditions and/or technologies, such as modifying time-temperature of frying or baking. Alternatively, reformulation, selections of cereal or potato varieties low in MRPs precursors, addition of glycine, cysteine, natural antioxidant or enzyme have been proposed as effective procedure in different products[6,8].

Since the discovery of acrylamide formation in heated food, the matter of the study has been increasingly deepened[6]. Bivalent and monovalent ions such as sodium chloride may influence MR development through the dehydration of various key intermediates. Gökmen and Şenyuva[10] demonstrated that the addition of polyvalent cations such as Ca^{2+} prevents acrylamide formation in asparagine-glucose model system, but at the same time the formation of HMF and furfurals was significantly increased. Levine et al.[11] in a dough model system found that acrylamide concentration decreased upon the increase of sodium chloride concentration. Carle et al.[12] showed that sodium chloride plays a dual role in acrylamide formation: at NaCl concentration from 1% to 2% acrylamide formation decreases significantly; instead, increasing salt concentration more than 2%led to an increase of acrylamide content. Gökmen and Şenyuva[10] in a liquid model system constituted by 10 µmol/l of asparagine and glucose, found that the final acrylamide concentration was higher for the control, declined for NaCl concentration from 0.5 to 5 µmol/l and significantly increased for NaCl concentration from 5 to 20 µmol/l.

On the other hand, the HMF formation is more clearly linked to NaCl concentration: monovalent cations favored the dehydration of key intermediates leading to the increase of HMF formation[10]. This hypothesis is widely demonstrated both in commercial dextrose preparations[14] and in biscuits model system[17]in the presence of 0.45% and 0.7% of NaCl respectively.

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Ingredients encapsulation is characterized by the incorporation of bioactive molecules in small capsules that release their contents at controlled rates over prolonged periods of time[15]. Encapsulation in foods has been already used to avoid the degradation of bioactive compounds: coating allowed the physicochemical protection of functional molecules and ingredients. Vitamins, antioxidant, flavorings and preservatives have been incorporated using appropriate coating according to the desired functionality[16].

Encapsulation can be a smart approach to limit the contact between reactants in food matrix, so it can be used to modulate the Maillard Reaction during thermal treatment.

In this paper the use of encapsulation to prevent the formation of undesired MR products was investigated. As the effect of salt in promoting HMF formation was well established in different systems[13-14,17] , NaCl encapsulation was selected to verify the efficacy of encapsulation strategy to prevent HMF formation in foods. To this purpose differently coated NaCl preparations were added in the formulation of biscuits monitoring the formation of HMF and acrylamide at different cooking times. Results demonstrated that encapsulation of NaCl significantly decreased HMF formation without affecting color or sensorial properties of the biscuits.

Materials and methods

Chemicals

Acetonitrile, water and methanol for HPLC and LC/MS/MS determination andsodium chloridewere obtained from Merck (Darmastadt,Germany). Formic acid (98%) was purchased from J.T. Baker (Deventer, Holland).Acrylamide,[2,3,3-d_3]-acrylamide, 5-hydroxymethylfurfural (HMF)standards and sucrose were purchased from Sigma (St. Louis, MO).All the samples were filtered through nylon filters25 mm 0.45 µm and 2.5 ml conventional syringes (BD, Franklin Lakes, NJ) equipped with a PTFE adapter (Phenomenex, Torrance, CA). Carrez reagent potassium salt and Carrez reagent zinc salt were purchased from Carlo Erba (Milano, Italy).

Encapsulation process

Microencapsulation by spray-coating was performed using a UNI-GLATT device in a bottom spray configuration (Glatt, Germany). Sodium chloride (Labogros, Saint Herblain, France) was coated with three different materials: a melted fatty acid blend (stearic acid Tp 18/55, MP = 55 °C) candelilla wax (MP = 70 °C) and carnauba wax (MP = 85 °C). All the materials were purchased from Interchimie, Bobigny, France). Spray-coating parameters were adjusted to obtain an homogeneous coating. The coating material was sprayed through a nozzle onto fluidized sodium chloride. Droplets progressively covered sodium chloride surface to achieve a uniform coating after

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few minutes of operation.

Blank microparticles were produced by spray-cooling. The material (stearic acid Tp 18/55, candelilla wax or carnauba wax, indicated as SPAB, CanW and CarW respectively) was melted and heated above melting temperature. Melted material was distributed on a spinning disk by a peristaltic pump. Micro-droplets were ejected by centrifugal force and solidified by air-cooling (room temperature) to produce solid microparticles.

Melting point measurement

The melting points of encapsulated salt and its respective empty coating material were measured using a TA Q20 model DSC apparatus (TA Instruments, New Castle, DE). The apparatus was calibrated with indium (melting point = 156.6^{o}C, ΔH=28.5 J/g). The DSC runs were operated under nitrogen gas atmosphere (30 ml/min) using an empty pan as the reference. After approximately 2 mg of material was weighed, the aluminium pan was hermetically sealed. The pan was placed into the apparatus and scanned over an appropriate temperature range at a heating rate of 5°C/min.

Biscuit samples

Model cookies were prepared according to a recipe described in AACC (American Association of Cereal Chemists) method 10–54 (AACC, 2000) with some modifications. Refined flour and shortening were kindly supplied by Kraft Foods (Glattpark, Switzerland), while ammonium bicarbonate and sodium bicarbonate were purchased from Sigma-Aldrich. The model biscuits were prepared according to thirteen recipes as summarized in Table 1. The ingredients were mixed in order to evaluate the specific effect of salt concentration and salt encapsulation on HMF formation. To achieve the maximum homogeneity among the various batches, each dough was rolled between two bars with the height of 3 mm and were shaped in a disk of 30 mm diameter.

Biscuits were baked at 200°C for 13 min in a forced air circulation oven (Memmert, Schwabach, Germany).The cooking protocol was designed to ensure the same thermal treatment for the biscuits. The position of the tray was always the same for the various batches and six biscuits were always placed in the same position.. After cooking the different recipes were introduced in polystyrene box to avoid any external interference.

Preparation of model systems

A model system composed of sucrose and NaCl was used to determine the effect of salt on HMF formation. A total of 10 µmoles of sucrose and NaCl were transferred to 25 ml test tube (Pyrex, 25 ml) as their aqueous solutions. Total reaction volume was adjusted to 100 µl with deionized water. A total of 300 mg of silica gel was added to cover the reaction mixture and the tube was tightly closed with a screw cap. The reactions were performed in an oil bath at 200^{o}C for 5, 10 and 20 min. All reactions were performed in triplicate. The reaction mixtures after heating were suspended in 2

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ml of 10 mM formic acid and the aqueous extract was obtained by vortexing the tube for 2 min. After centrifugation at 11180 g for 5 min, 1 ml of the supernatant was passed through a 0.45 µm nylon syringe filter into a vial.

Physical analysis of microparticles

a) Particle size distribution

Microparticles size distributions were determined by laser granulometry (Malvern Mastersizer, Malvern UK). Samples were analyzed in triplicate, 5000 particles were counted for each analysis.

b)Optical microscopy

Microparticles pictures were obtained with a motic binocular microscope (magnification x 30) equipped with a SMZ motic camera (motic, Germany).

c) Scanning electron microscopy (SEM)

The microparticles morphology was observed by Scanning Electron Microscopy (SEM, JSM 5800, Jeol, Japan). The microparticles were mounted on a support platform (9 mm diameter) with a conductor adhesive (Carbon tabs, Agar Scientific) and fixed with a thin carbon layer (ca. 20 nm). To make material conducting, the sample was covered with a fine layer of gold (step of metallization). The pressure was set to10^{-3} mPa during the metallization and to $6{\cdot}10^{-6}$ mPa inside the apparatus.

d) Conductimetry

Conductivity experiments were carried out using two different devices. Sodium chloride content was measured using a WTW 315i conductimeter equipped with a TetraCon 325 probe (WTW, Germany). Microparticle samples were dispersed in water and crushed prior to titration. All measurements were performed in triplicate. Sodium chloride cumulative release in water was followed with a T50 titrator equipped with an Inlab 730 probe and an internal agitation system (Mettler Toldeo, France). Microparticle samples were loaded into the conductimeter cell containing 80 ml of deionized water. The release of sodium chloride was monitored over 20 min under agitation.

HMF analysis

HMF was determined according to the method of García-Villanova, Guerra-Hernández, Martinez Gómez and Montilla[18]with minor modifications. The biscuits were ground in a knife mill Grindomix 200 (Retsch, Haan, Germany). One g of powder was weighed;10 ml of deionised water were added in a 15 ml centrifuge tube along with 500 µl of Carrez reagent potassium salt and 500 µl of Carrez reagent zinc salt. The tubes were shaken vigorously for 1 min. The resulting mixture was centrifuged at 2700gfor 10 min at 4°C. The supernatant was then collected in a 20 ml volumetric flask and two further extractions were performed using 5 ml of deionised water. Finally the pellets were discarded and the supernatants were filtered through a 0.45 µm nylon filter; 2 ml

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were collected and used for HPLC analysis. The HPLC system consisted of LC-10AD class VP pumps and a SPD-M10A diode array detector equipped with a SCL-10A class VP controller, all from Shimadzu (Kyoto, Japan). The mobile phase was a mixture of acetonitrile in water (5% v/v) at a flow rate of 1 ml/min under isocratic conditions and a Synergi 4µmHydro-RP 80Å, 250 x 4.6 mm column (Phenomenex, Torrance, CA) was used for the chromatographic separation. The UV detector was set at 280 nm and HMF was quantified using the external standard method. A calibration curve was built within the range 0.1 – 10 µg/ml and the coefficient of determinationr^2 was 1 after three replicates. The limit of detection (LOD) was 0.050 µg/ml, while the limit of quantification (LOQ) was 0.150 µg/ml. All the analyses were performed on quadruplicate by injecting in the system 20 µl of the biscuits extracts and the results expressed as µg/g of sample.

Acrylamide analysis

Acrylamide was measured following the same extraction protocol used for HMF determination and after the grinding of the biscuit samples 100µl of internal standard [2,3,3-d_3]-acrylamide (20 µg/ml in order to achieve a final concentration of 90 ng/ml) were added[19]. Two ml of supernatants were collected and passed through an Oasis HLB - cartridge (Waters, Milford, MA) previously activated with 1 ml of methanol and 1 ml of deionized water; the first eight droplets of the effluent were discarded in order to prevent any dilutions of the sample and the following droplets were collected and 20 µl of the final test solution were injected onto LC column for quantification by LC/MS/MS. Identification and quantitative determination of acrylamide and [2,3,3-d_3]-acrylamide was carried out using an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA) coupled to a Turboionspray (TIS) interface, equipped with an HPLC binary micropumps Series 200 (Perkin Elmer, USA). Chromatographic separation of acrylamide and [2,3,3-d_3]-acrylamide was achieved through an Inertsil column 25x0.46 cm, 5µm (GLSciences, Torrance,CA) and the following gradient elution was applied: 0–3 min 100% A, 3–8 min 93% A, 8–12 min 100%, at a flow rate of 0.800 µl/min, whereof 200µl were split into the ion source.

The quantification was carried out in MRM (Multiple Reaction Monitoring) at *m/z* ratios of 72 and 75 for acrylamide and [2,3,3-d_3]-acrylamide, respectively. Specific molecular fragments corresponding to 55 and 44 *m/z* and 58 and 44 *m/z* were also monitored. The daughter ions were obtained through fragmentations with the following specific conditions: the source temperature was set at 350°C,nitrogen was used as nebulizer gas at flow rate of 12 l/h and the needle and cone voltages were set at 3.0 kV and 100 V, respectively. Under the above – mentioned chromatographic conditions the acrylamide and its corresponding isotope standard eluted at 7.4 min. Acrylamide was quantified using a linear calibration curve built with specific solutions of acrylamide and [2,3,3-d_3]-acrylamide dissolved in water (50-500 ng/ml). The LOD and the LOQ were respectively10 ng/ml

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and 30 ng/ml for acrylamide and the coefficient of determination $r^2$was 0.9998. The internal standard was used for the recovery test and it varied from 95 to 103%. All the analysis were performed in quadruplicate and the results expressed as ng/g of sample.

High Resolution Mass Spectrometry Analysis (HRMS) of reaction products formed in model system

Extracts of model systems were analyzed by HRMS in order to identify the reaction intermediates and products. A Thermo Scientific Accela UHPLC system (San Jose, CA) coupled to a Thermo Scientific Exactive Orbitrap HRMS was used. The HRMS system was operated in positive electrospray ionization mode. The chromatographic separations were performed on Atlantis T3 Column (250 mm x 4.6 mm id; 5 cm) (Waters Corporation, Milford, USA) using 0.05% aqueous formic acid and methanol isocratically (70:30)at a flow rate of 0.5 mL/min (30^{o}C) for 15 min. The scan analyses were performed in an *m/z* range between 50 and 600 at ultra-high resolving power (R=100.000). The data acquisition rate, the automatic gain control target and maximum injection time were set to 1 Hz, 1x10^6 and 100 ms, respectively. The source parameters were as follows: sheath gas flow rate 45 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units), sweep gas flow 3 (arbitrary units) spray voltage 3 kV, capillary temperature 300^{o}C, capillary voltage 25 V, tube lens voltage 55 V and vaporizer temperature 300^{o}C. To confirm the reaction path leading to HMF, possible forms of sucrose decomposition products were extracted from the total ion chromatograms.

Sensory analysis

In order to determine if there was a detectable difference among the biscuits with different recipes, a triangle test was undertaken according to UNI ISO 4120 Norm[20]. Two sessions of 30 untrained panelists were conducted at 20-22°C in an eight booth sensory panel room equipped with white fluorescent lighting. The various samples were put in different vessels that were coded uniformly, using three digit numbers chosen at random for each test in order to prevent stimulus errors, logical errors or suggestion effect. In each session, panelist were asked to complete the test on two types of biscuits that consisting of two samples with 0.65% of sodium chloride and one at the same encapsulated sodium chloride concentration, as odd samples. The panelist were asked to taste the samples and decide which samples was different from the others; in the first session they evaluated the general differences and in the second session they were asked to decide the differences in salty taste. For the two sessions a forced-choice procedure was used. The level of significance was □ 0.01. It was decided to evaluate if any difference was detectable in the samples.

Statistical analysis

Data were analyzed by ANOVA and means were compared by Duncan’s Multiple Range Test.

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Results and discussion

Sodium chloride (mean diameter 556 µm) was successfully encapsulated using the fluidized-air bed coating. Microparticles with mean diameters of 722, 720 and 716 µm were obtained for stearic/palmitic acid blend (SPAB), candelilla wax (CanW) and carnauba wax (CarW) coating respectively. Sodium chloride content was measured by conductimetry after crushing and total release in water and was titrated at 877 ± 8; 752 ± 10; and 774 ± 13 mg/g of microparticles(for stearic/palmitic acid blend, candelilla wax and carnauba wax coating respectively). As showed in **Figure 1** (top panels) encapsulated sodium chloride microparticles present intermediate shapes from cubic (sodium chloride typical shape, thin coating) to spherical (thick coating). Only few microparticles present poor or no absence of coating. Microparticles surface morphology was analysed by scanning electron microscopy (SEM). SEM images highlight the differences of the surface roughness: particles coated with caranauba wax revealed a very smooth surface compared to those coated with candelilla wax or stearic/palmitic acid blend.

The barrier ability of the coating to isolate sodium chloride from external environment was firstly evaluated by conductimetry. Microparticles were dispersed in water and the release of sodium chloride was monitored over the time plotting the increase of water conductivity. Blank microparticles made of coating material showed no significant modification of water conductivity (data not shown). Results with coated sodium chloride microparticles showed in **Figure 2** demonstrated that carnauba coating had the slowest release compared to candelilla wax coating or steraric/plamitic acid blend coating.

The differences among carnauba wax (CarW), candelilla wax (CanW) and stearic/palmitic acid blend (SPAB) coating were due to the homogeneity of the coating. The more homogeneous the coating was, the less the sodium chloride was released. Roughness and heterogeneous coating structure that appeared on SEM pictures could allow a better water access inside the microparticles and a faster release of sodium chloride.

NaCl encapsulation was already reported in the literature mainly to verify the release of the oil electrolytes in water emulsion systems[21,22]; however its use to modulate the development of chemical reactons in foods was never proposed thus far. In this case the slow melting of coating during biscuits coating might reduce the participation of NaCl to chemical reactions and, at the same time, should guarantee its presence at the end of the cooking.

In Figure 3 the effect of NaCl concentration on the formation of HMF and acrylamide in biscuits was reported. Data clearly showed that NaCl promoted the formation of HMF in biscuits. This result confirmed those previously obtained in model systems[11] and for the first time demonstrated that the presence of 0.65% NaCl, which is the standard concentration of salt used in many

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commercial biscuits, increase the formation of HMF up to 75%. NaCl showed a specific effect towards HMF, while the formation of acrylamide was not significantly modified by the presence of salt. Biscuits with 0.65% (0.109 mol/kg) of NaCl, showed an average acrylamide concentration of 0.278 μg g^{-1} ± 0.040, while the control without NaCl had the highest concentration: 0.313 μg g^{-1} ± 0.057. This data confirmed previous studies already mentioned in the introduction showing that there is not a direct relationship between NaCl concentration and acrylamide formation[11-13].

The mechanisms leading to conversion of sucrose into HMF through the fructofuranosyl cation at high temperatures have been previously described[23]. As shown in **Figure 4**, both glucose and fructofuranosyl cation can generate HMF by the elimination of two and three moles of water, respectively. The model sucrose system heated at 200^{o}C formed HMF with an initial rate of 1.11 nmol min^{-1}. With NaCl, the rate of HMF formation from sucrose increased to 8.13 nmol min^{-1}. (**Figure 5**).This confirmed the catalytic role of sodium on the pyrolysis of sucrose leading to HMF. It was a fact that the presence of NaCl accelerated the pyrolytic decomposition of sucrose during heating at 200^{o}C. The rate of sucrose decomposition increased from 2.85 μmol min^{-1} to 10.18 μmol min^{-1} when NaCl was present in the reaction mixture during heating. It is thought that NaCl as a metal cation acts as Lewis acid in the reaction mixture that accelerates the decomposition of sucrose. It has been previously shown that dehydration of hexoses is catalyzed by organic acids, inorganic acids, salts, and Lewis acids[14,23].

Formation of key intermediates in the heated model reaction mixtures was monitored to understand better the role of NaCl in sucrose decomposition in a semi-quantitative way. Scan HRMS analyses of sucrose pyrolyzates with and without NaCl tentatively confirmed the presence of 3-deoxyglucosone, 3,4-dideoxyosone, together with HMF having m/z of 163.0601, 145.0495 and 127.0390, respectively, with a very high mass accuracy (Δ<2.0 ppm). Extracted ion chromatograms of these compounds in the pyrolyzate of sucrose heated with NaCl at 200^{o}C for 10 min are shown in **Figure 6.** The rates of the formation of 3-deoxyglucosone and 3,4-dideoxyosone from sucrose increased by a factor of 4.3 and 23.5 times in the presence of NaCl during heating as shown in **Figures7a** and **7b**.

To verify the effectiveness of the encapsulation strategy to mitigate the formation of potentially harmful compounds the HMF was an appropriate target and the NaCl a suitable reagent to be modulated by encapsulation. To prevent NaCl participation to the dehydration of key intermediates for HMF formation, NaCl should be coated using a material which melt near to the end of biscuits cooking time. In fact, at the end of the cooking time NaCl should be free because of the sensorial need to have some salty sensation in the biscuits.

The effect of using NaCl encapsulated ingredients on HMF formation is shown in **Figure 8.**Using

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three different coating materials the encapsulation of NaCl led in all cases to a significant inhibition of HMF formation. Carnauba wax proved to be the most effective coating, giving an HMF concentration comparable to that of biscuits without NaCl. In particular HMF reduction was 18%, 41% and 61% for stearic/palmitic acid blend, candelilla wax and carnauba wax coating , respectively.

The results of this experiment fully confirmed the correctness of the proposed strategy: subtracting to the reaction an agent catalyzing the HMF formation is an effective mitigation strategy to prevent its formation and in this respect encapsulation proved to be a very effective tool.

Analysis revealed that the heat resistance of the coating is inversely correlated with the HMF formation, thus confirming that coating melting point was a key factor to determine the final HMF concentration. Blocking NaCl inside the microparticles reduce the time of its participation to the reaction converting sucrose into HMF. The increase of the melting point of the coating delays sodium chloride release and reaction during the oven baking. Regarding the cooking of the biscuits, the coating quality plays an important role either. An homogeneous coating decreases water acces to sodium and finally sodium chloride release and reaction. The melting point of the coating is inversely correlated with amount of HMF formed in biscuits during baking ($y=-3.3119x+358.87$) with a correlation coefficient of 0.931.Obviously, encapsulating material with higher melting point kept NaCl inside the microparticles preventing its catalytic action on the formation of HMF from sucrose.

The baking conditions determine the complete melting of microparticles coating at the end of the cooking time. This was confirmed by the sensory analysis results summarized in **Table 2a** and **2b**. A panel of untrained consumers was not able to distinguish the biscuits manufactured with free NaCl by those obtained with encapsulated salt. In fact, triangle test failed to rich the threshold of minimum correct answer. So the sensory study confirmed that that encapsulated sodium chloride recipes were not perceived as different from that containing free 0.65% sodium chloride

In conclusion, this is the first paper demonstrating that the encapsulation of some ingredients can be used to prevent the formation of undesired MRPs products in thermally treated foods.

Abbreviations used

MRPs: Maillard Reaction products; HMF: 5-hydroxymethylfurfural;HRMS: high resolution mass spectrometry; SPAB: Stearic palmitic acid blend; CanW: Candelilla wax; CarW: Carnauba wax.

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Table 1: Recipes used to prepare cookies. The following ingredients were common for all recipes: wheat flour, 40 g; sucrose, 17.5 g; Shortening, 10 g; deionized water, 8.8 g; sodium bicarbonate, 0.4 g; ammonium bicarbonate, 0.2 g; SPAB: stearic/palmitic acid blend; CanW: Candelilla wax; CarW: Carnauba wax.

Amount in the dough (g)			
Recipes	NaCl	Coated NaCl microparticles	Coating materials microparticles
NaCl 0 %	0	/	/
NaCl 0.32%	0.250	/	/
NaCl 0.65%	0.500	/	/
NaCl 1%	0.775	/	/
Encapsulated NaCl (SPAB)	/	0.600	/
(SPAB) Microparticle + NaCl	0.500	/	0.100
(SPAB) Microparticle	/	/	0.100
Encapsulated NaCl (CanW)	/	0.610	/
(CanW) microparticle + NaCl	0.500	/	0.110
(CanW) microparticle	/	/	0.110
Encapsulated NaCl (CarW)	/	0.625	/
(CarW) microparticle + NaCl	0.500	/	0.125
(CarW) microparticle	/	/	0.125

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Table 2: Triangle test results on biscuits. SPAB: stearic/palmitic acid blend; CanW: Candelilla wax; CarW: Carnauba wax. Number of correct/incorrect responses for the general differences (2a) and for salty taste (2b). The panelist were asked to identify any differences between the two samples; [illegible] was set at 0.01. The minimum number of correct responses needed to conclude that two samples are similar, based on a triangle test, was 17; *significant.

Table 2a

2a			
Biscuits served as odd sample	No. of assessors	No. of correct/incorrect judgments	Minimum correct answer
Encapsulated NaCl CanW	30	12/27	17*
Encapsulated NaCl CarW	30	11/24	17*
Encapsulated NaCl SPAB	30	9/28	17*

2b			
Biscuits served as odd sample	No. of assessors	No. of correct judgments	Minimum correct answer
Encapsulated NaCl CanW	30	9/26	17*
Encapsulated NaCl CarW	30	9/26	17*
Encapsulated NaCl SPAB	30	11/24	17*

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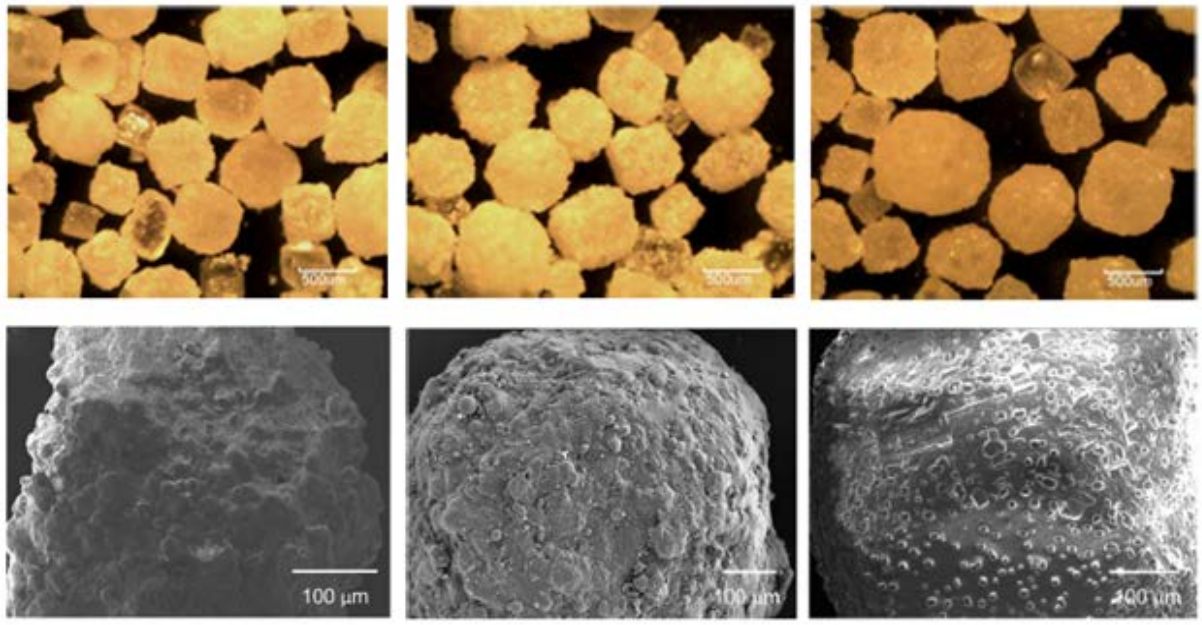

Figure 1: NaCl microparticles pictures from optical microscopy (top) and SEM (bottom). Microparticles coated respectively with stearic/palmitic acid blend (SPAB), candelilla wax (CanW) and carnauba wax (CarW) are presented from left to right. The images obtained from optical microscopy showed the general aspect of the microparticles while SEM images showed the surface aspect of the NaCl microparticles

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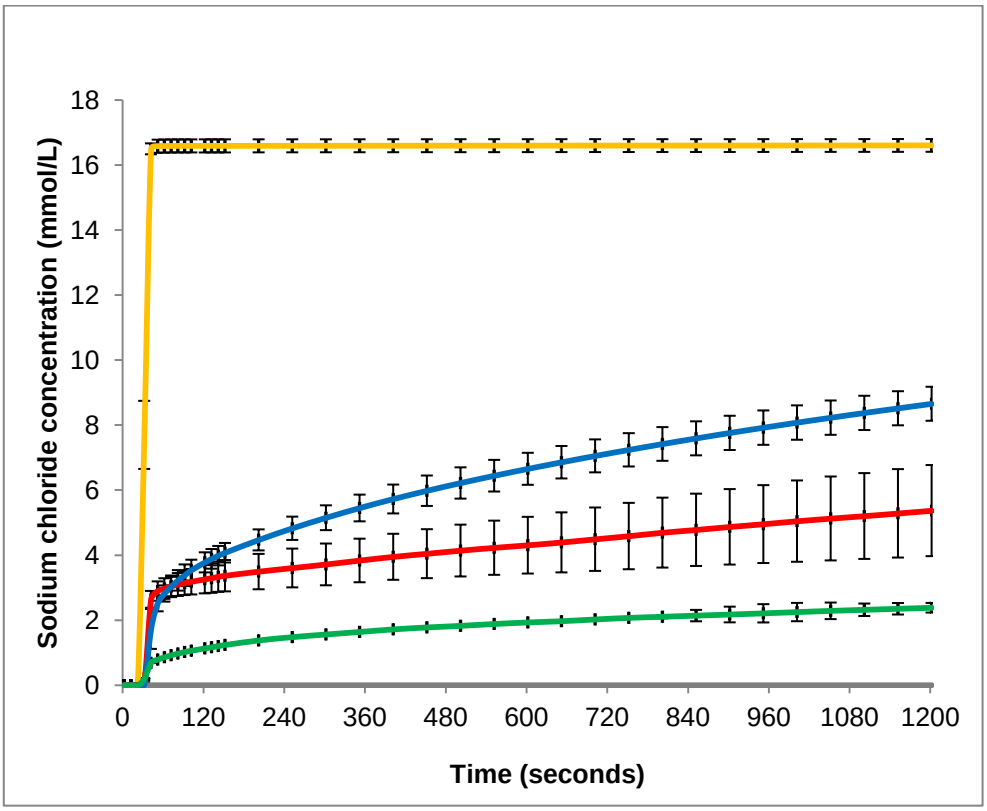

Figure 2: Sodium chloride cumulative release profiles in water (standard deviation on triplicate analysis), grey: distilled water; yellow: pure NaCl; blue: NaCl coated with candelilla wax (CanW); red: NaCl coated with stearic / palmitic blend (SPAB) and green: NaCl coated with carnauba wax (CarW)

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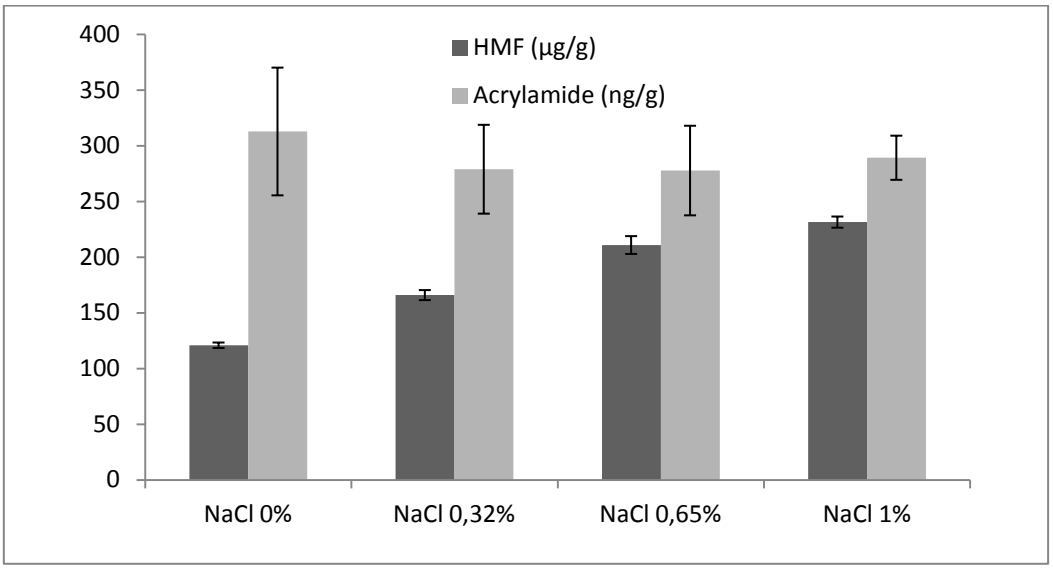

Figure 3: Black bars: concentration of HMF (µg/g); Light grey bars: concentration of acrylamide (ng/g) in biscuits containing increasing amount of NaCl (refers to Table 1 for the formulations).

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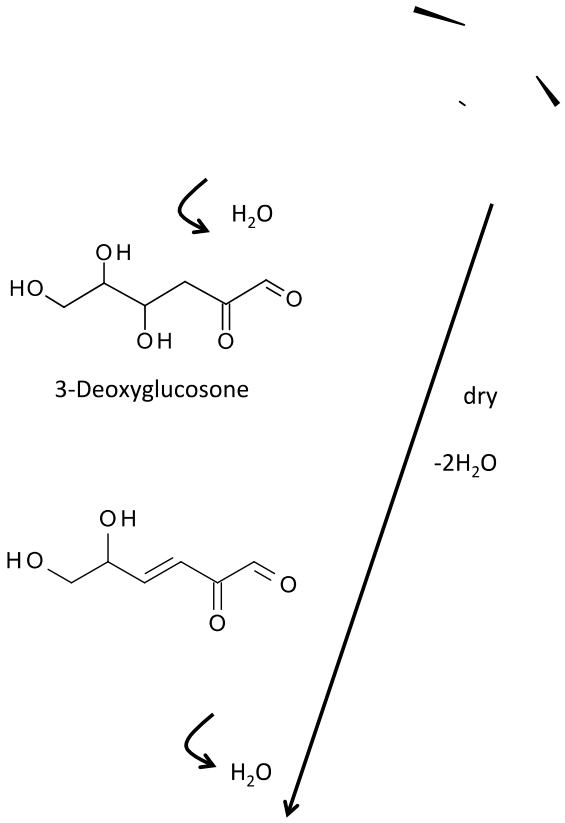

Figure 4: Sucrose pyrolysis pathway. (Adapted from Perez Locas and Yaylayan [23])

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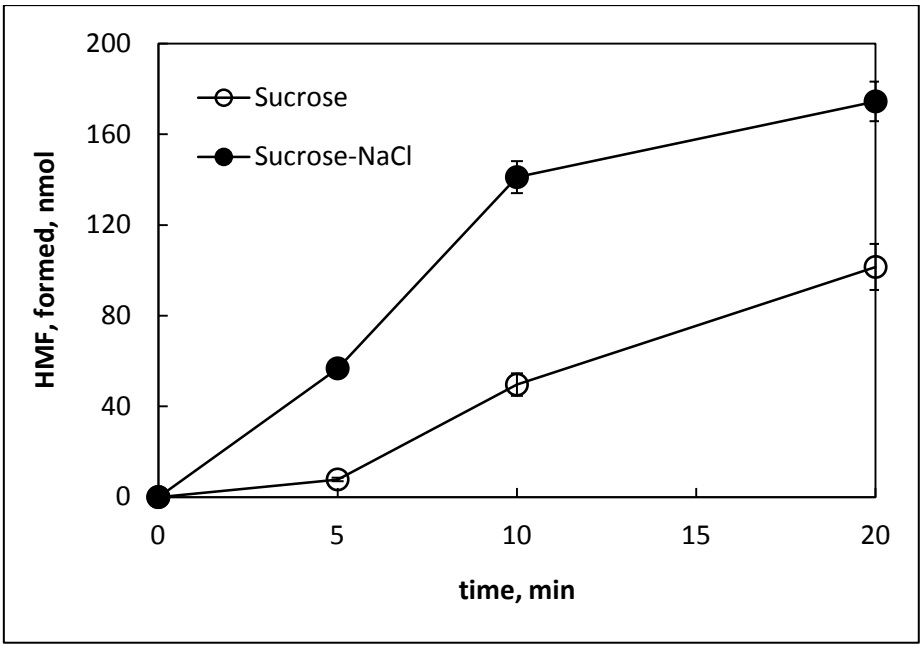

Figure 5: Amount of HMF formed during heating sucrose with and without NaCl at different time points

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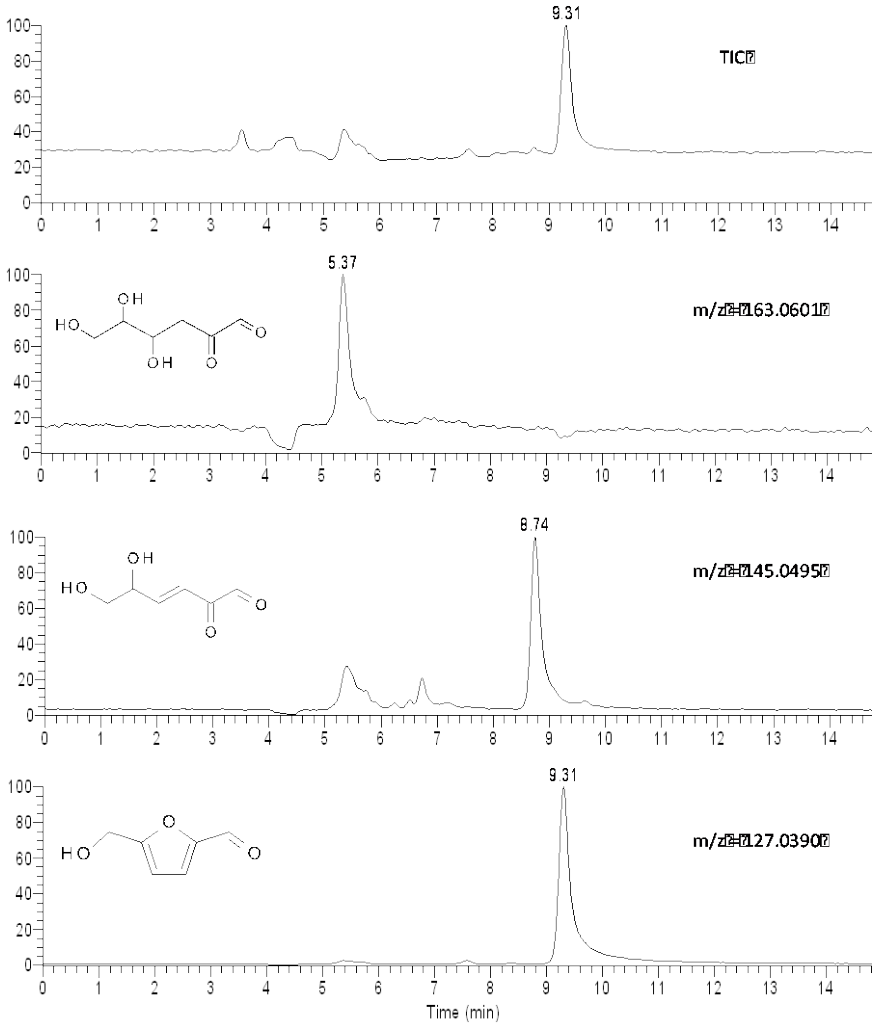

Figure 6:Extracted ion chromatograms of 3-deoxyglucosone, 3,4-dideoxyosone, and HMF formed in the model system heated at 200oC for 10 min

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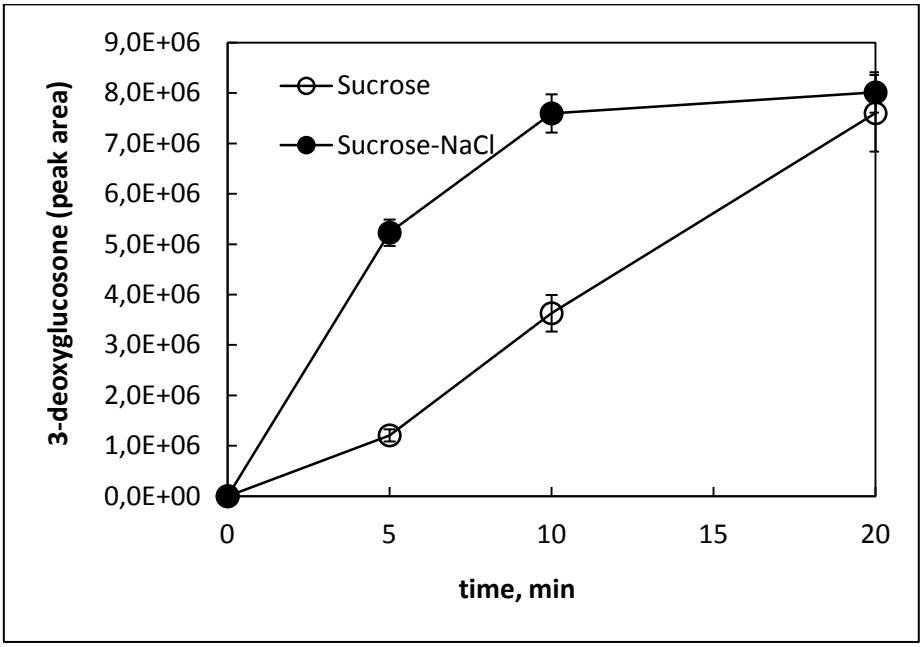
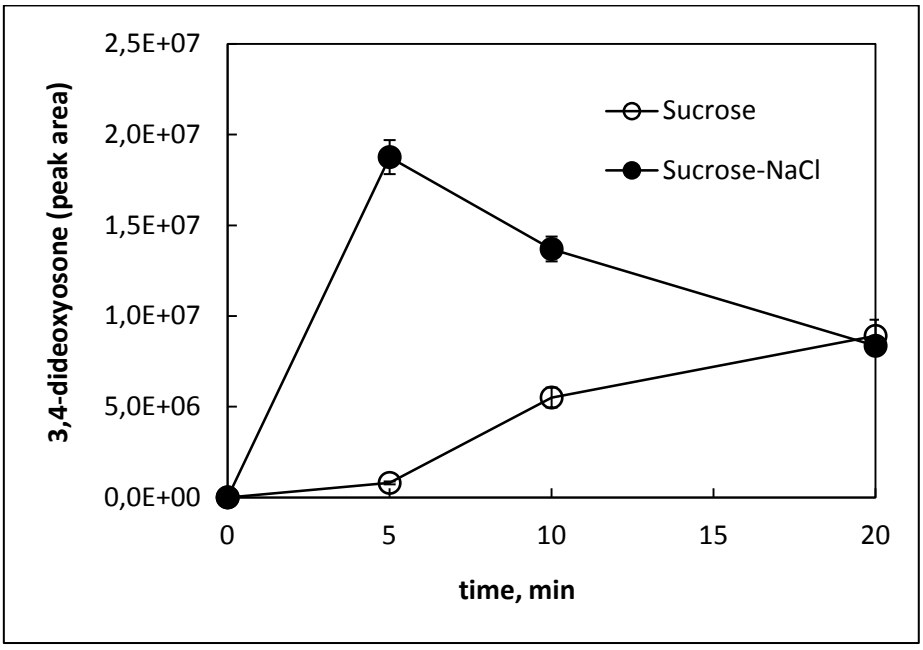


(a)



(b)

Figure 7: Amounts of (a) 3-deoxyglucosone, and (b) 3,4-dideoxyosone formed during heating sucrose with and without NaCl at different time points

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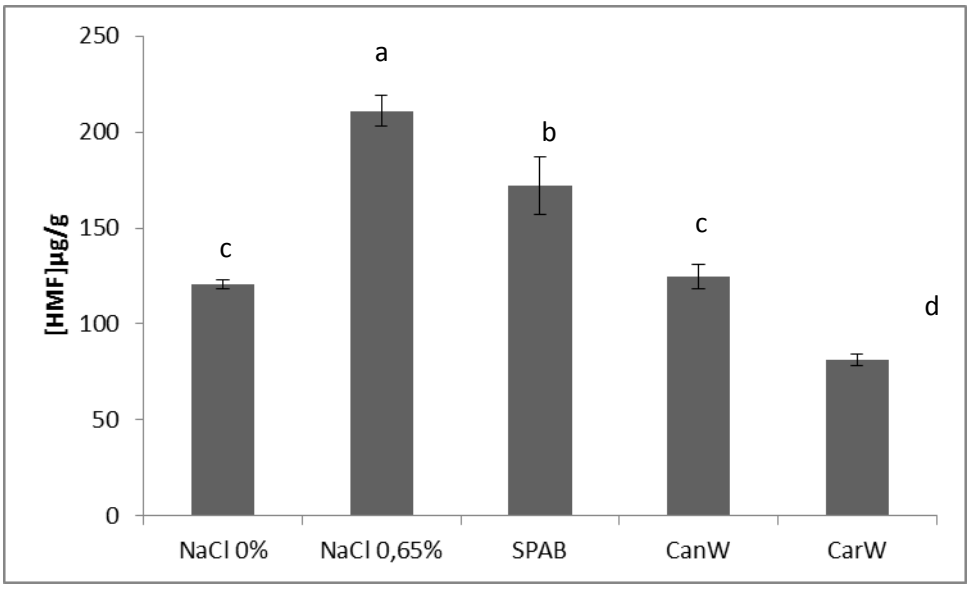

Figure 8: HMF concentration in biscuits prepared with different types of encapsulated NaCl. All samples have the same amount of NaCl (0.5 g). SPAB: encapsulated NaCl with stearic/palmitic acid blend coating; CanW: encapsulated NaCl with candelilla wax coating; CarW: encapsulated NaCl with carnauba wax coating. The significant differences among HMF content were determined by Anova analysis and Duncan's multiple range test ($p \leq 0.05$).

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A.3

Role of Curcumin on the Conversion of Asparagine into Acrylamide During Heating

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Abstract

This study aimed to investigate the ability of curcumin to convert asparagine into acrylamide during heating at different temperatures. Binary and ternary model systems of asparagine-curcumin and asparagine-curcumin-fructose were used to determine the role of curcumin on acrylamide formation in competitive and uncompetitive reaction conditions. The results indicated that curcumin could potentially contribute to acrylamide formation under long term heating conditions as long as asparagine is present in the medium. The amount of acrylamide formed in the ternary system was slightly higher than in the binary system during heating ($p<0.05$), because of the higher concentrations of carbonyl compounds initially available. The kinetic trends were similar in both model systems evidencing that fructose reacted with asparagine more rapidly than curcumin. The data revealed that acrylamide formation in a temperature range of 150-200^{o}C obey Arrhenius law with activation energy of 79.1 kJ/mole. Data of this work showed the possibility that antioxidants having a carbonyl compound can react directly with ASN leading to acrylamide. The addition of antioxidants to foods might increase the formation of acrylamide upon long term heating if free sugar concentration is low and ASN concentration is relatively high.

Keywords: Curcumin, asparagine, acrylamide formation, Maillard reaction

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Introduction

In 2002, Swedish researchers have first reported the formation of acrylamide in foods processed at elevated temperatures (Tareke et al. 2002). Presence of acrylamide in common heated foods has been considered an important food safety issue by international authorities. Acrylamide has been classified as probable carcinogenic to human by the International Agency for Research on Cancer (IARC 1994).

Several researchers have established that the main pathway of acrylamide formation in foods is linked to the Maillard reaction and to the presence of free asparagine (ASN)(Mottram et al., 2002; Stadler et al., 2002; Stadler et al., 2004; Zyzak et al., 2003). Studies to date clearly show that ASN is mainly responsible for acrylamide formation in heated foods after condensation with a carbonyl source. Zyzak et al., (2003) have investigated the effectiveness of different carbonyls in promoting the conversion of ASN into acrylamide. They have found that carbonyl compounds such as 2-deoxyglucose, ribose, glyceraldehyde, glyoxal, and decanal generated acrylamide from asparagine. Some of the Maillard reaction products also have reactive carbonyls capable of forming Schiff base with asparagine and thus producing acrylamide (Amrein et al.2006; Koutsidis et al. 2008). Stadler et al. (2002) have studied the reactivity of various carbonyl and hydroxycarbonyl compounds in comparison with glucose. Glyoxal has been reported as the most reactive dicarbonyl compound in yielding acrylamide from ASN while, among lipid oxidation product, also 2,4-decadienal was very effective (Zamora et al. 2010a).

The role of antioxidants, particularly phenolic compounds in preventing acrylamide formation was largely investigated, but data from the various studies are discordant (Zhang and Zhang 2008; Brat et al. 2010; Kotsiou et al. 2011). Several studies have reported that phenolic compounds prevent acrylamide formation while in others no effect or even an increase of acrylamide concentration was found (Napolitano et al., 2008; Corkeet al. 2009; Ou et al. 2010). This was attributed to the ability of the antioxidant compounds to react directly with acrylamide precursor, with acrylamide after its formation or with intermediates of the reaction.

It is a fact that certain phenolic compounds bear carbonyl group that may compete with the carbonyl group of reducing sugars in a Maillard type reaction during heating. Owing to its carbonyl group, curcumin can be expected to react with ASN yielding acrylamide under certain heating conditions. Beside its antioxidant property, curcumin is widely used in various foods up to 0.5 g/kg such as fats and oils, emulsions, confectionery, dairy products, cereal products, meat and fish products, spices and sauces as a natural coloring agent (FAO, 2004). This study aimed to investigate the role of curcumin on acrylamide formation during heating at elevated temperatures. Competition of curcumin with reducing sugars was investigated in model and in crust-like food systems.

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Materials and Methods

Chemicals and consumables. Acrylamide (99%) was purchased from Sigma (Deisenhofen, Germany). $^{13}C_3$-acrylamide (99% isotopic purity) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).3-Aminopropionamide(3-APA) hydrochloride (97%) was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany). L-Asparagine (98%) and silica gel were purchased from Merck (Darmstadt, Germany). Curcumin (79% curcumin, 19% desmethoxycurcumin and 2% bisdesmethoxycurcumin) was purchased from Sigma (Deisenhofen, Germany) Formic acid (98%), acetonitrile, and methanol (HPLC grade) were purchased from J. T. Baker (Deventer, Holland). Syringe filters (nylon, 0. 45 µm), Oasis MCX (1 mL, 30 mg) and Oasis HLB (1 mL, 30 mg) solid phase extraction cartridges, Acquity UPLC HSS T3 column (100 x 2.1 mm i.d., 1.8 µm), Atlantis T3 column (250 x 4.6 mm i.d., 5 µm) were supplied by Waters (Millford, MA, USA). Stock solution of acrylamide was prepared in water to a concentration of 1 mg/mL. Working solutions were prepared by diluting the stock solution with water to concentrations of 1, 5, 10, 20, 50 and 100 ng/mL for acrylamide.

Preparation of samples

Model system. Aportion (0.1 mL) of the solution containing 0.01 mmol of ASN alone or a binary mixture of carbonyl compound and ASN (0.01 mmol each) were transferred to a glass tube containing 50 mg of silica gel. Curcumin (CUR) and fructose (FRU) were used as carbonyl compound in model systems. Then, 300 mg of silica gel was added to cover the reaction mixture, and the tube was tightly closed with screw cap. The reactions were performed in oil bath at 150, 180 and 200°C for 5, 10, 20, 30 and 60 min in order to obtain kinetic and thermodynamic data for acrylamide formation. All reactions were performed in triplicate, and mean values were reported. Model

Crust model. Crust model samples were prepared according to method described by Acar and Gokmen (2009) with some modifications. Bread dough was prepared by mixing 20 g of wheat flour with 11.4 mL of water. In the crust model, glucose (GLC) was chosen instead of FRU, because it is the main form of simple sugars in wheat flour also deriving from starch hydrolysis. After the addition of 1% curcumin or GLC, the dough samples were rolled out to obtain disks having a diameter of 3 cm. Disk weights were adjusted to 0.5 g to ensure a fixed value of dry matter in the samples. The average thickness of the disks was 0.5 mm. The disks were baked in the oven (Memmert UNE 400, Germany) at 180˚C up to 30minto obtain bread crust resembling samples. The samples were kept at -18^{o}C prior to analysis. Baking trials were performed in triplicate, and mean values were reported.

LC-MS/MS analysis of acrylamide. Model system samples were spiked with 100 ng of $^{13}C_3$-

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acrylamide, and extracted with 10 mL of 10 mM formic acid by vortexing the tube for 2 min. Then, the extract was centrifuged at 11200 g for 5 min and supernatant was collected. In crust model, ground sample (0.25 g) was spiked with100 ng of $^{13}C_3$-acrylamide, and double extracted with 10mM formic acid (2.5 mL and 2.5 mL) by vortexing for 3 min. The combined extract was clarified by Carrez clarification. After centrifugation at 10000 g for 5 min, supernatant was collected. At the end of second extraction, collected supernatants were combined with vortexing for 2 min. 2.0 mL of the supernatant was passed through a preconditioned Oasis MCX cartridge to clean up the extract. The first 8 drops were discarded and the rest was collected into an autosampler vial. A Waters Acquity H Class UPLC system coupled to a TQ detector with electrospray ionization operated in positive mode was used to analyze the extracts to quantify acrylamide. The chromatographic separations were performed on an Acquity UPLC HSS T3 column using 10 mM formic acid with 0.5% methanol as the mobile phase at a flow rate of 0.3 mL/min. The column equilibrated at 40°C and Waters ACQUITY FTN Autosampler was held at 10°C during the analysis. The electrospray source had the following settings: capillary voltage of 0.80 kV; cone voltage of 21 V; extractor voltage of 4 V; source temperature at 120°C; desolvation temperature at 450°C; desolvation gas (nitrogen) flow of 900 L/h. The flow rate of the collision gas (argon) was set to 0. 25 mL/min. Acrylamide was identified by multiple reaction monitoring (MRM) of two channels. The precursor ion $[M+H]^+$ 72 was fragmented and product ions 55 (collision energy of 9 V) and 44 (collision energy of 12 V) were monitored. The dwell time was 0.2 sec for all MRM transitions. Concentration of acrylamide in samples was calculated by means of a calibration curve built in the range between 1 and 100 ng/mL (1, 2, 5, 10, 20, 50, 100 ng/mL). The limit of detection and limit of quantitation of acrylamide for crust model samples were 3 ng/g and 10 ng/g, respectively.

HPLC analysis of sugar. Samples were extracted with 5 mL of deionized hot water by vortexing the tube for 1 min. After filtering by syringe filters (nylon, 0.45 µm), 2 mL was passed through a preconditioned Oasis HLB cartridge to clean up the extract. The first 8 drops of the eluent were discarded and rest was collected into an autosampler vial. Prepared extracts were analyzed by an Agilent 1100 HPLC system (Waldbronn, Germany) consisting of a quaternary pump, a Rheodyne 7125 injector and temperature controlled column oven, coupled to a refractive index detector (RID). Chromatographic separations were performed on a Shodex Sugar SH-1011 (8.0 x 300 mm, 7 µm) column using 0.01 mMH_2SO_4 as the mobile phase at a flow rate 0.7 ml/min. Column temperature was set at 50°C.

Analysis of 3-APA. 3-APA formed was extracted with 10 ml of distilled water by vortexing the tube for 2 min. After centrifugation at 11180 g for 5 min, 2 ml of the supernatant was passed through a 0.45 µm syringe filter and collected into an autosampler vial. An Agilent 1200 HPLC

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system (Waldbronn, Germany), consisting of a binary pump, an autosampler and a temperature controlled column oven, coupled to an Agilent 6130 MS detector, equipped with an electrospray ionization (ESI) interface was used to analyze the extracts for 3-APA. The MS detector was operated in positive ionization mode using the following interface parameters: drying gas (N_2) flow rate of 13 mL/min, nebulizer pressure of 40 psig, drying gas temperature of 350°C, capillary voltage of 4 kV, and fragmentor voltage of 100 eV. The analytical separation was performed on an Atlantis T3 column using an isocratic mixture of 10 mM formic acid: methanol (70:30, v/v) as the mobile phase at a flow rate of 0.8 ml/min (40°C). Data acquisition was performed in SIM mode. Presence of 3-APA was confirmed by comparing both mass spectra and retention time of corresponding pure standard. Concentration of 3-APA was calculated by means of a calibration curve built in the range between 10 and 100 ng/mL. Signal response of the precursor ion $[M+H]^+$ having *m/z* of 89 was used for quantitation while signal response of the compound specific ion having *m/z* of 72 was used for confirmation in selected ion monitoring mode.

High resolution mass spectrometry analysis of reaction intermediates and products. The ASN-CUR model system samples heated at 180^{o}C for different times were analyzed by using high resolution mass spectrometry to confirm the structures of reaction intermediates and products. Reaction intermediates were extracted with methanol-water mixture (50:50, v/v) by vortexing the tube for 2 min. After centrifugation at 11180 g for 5 min, supernatant was passed through a 0.45 µm syringe filter and collected into an autosampler vial. The measurements were carried out by using a Thermo Scientific Accela Liquid Chromatography System (San Jose, CA USA) coupled to a Thermo Scientific Exactive Orbitrap high resolution mass spectrometry (San Jose, CA USA) operated in positive electrospray ionization (HESI) mode. The chromatographic separations were performed on Atlantis T3 Column (250 mm x 4.6 mm id; 5 µm) (Milford, MA, USA) using a gradient mixture of 0.05% aqueous formic acid and methanol as the mobile phase at a flow rate of 0.5 mL/min (30°C). The mobile phase gradient was programmed as follows: 70% of methanol for 8 min, linear increase to 95% of methanol within 4 min, 95% of methanol for 4 min, linear decrease to 70% of methanol within 4 min. The scan analyses were performed in an *m/z* range between 50-500 at ultra-high resolving power (R=100.000) to determine certain reaction intermediates and products including Schiff base, decarboxylated Schiff base, 3-APA and acrylamide in the mixtures. The data acquisition rate, the automatic gain control target, and maximum injection time were set to 1 Hz, $1x10^6$, and 100 ms, respectively. The HESI source parameters were as follows: sheath gas flow rate 30 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), discharge voltage 4.5 kV, discharge current 5 µA, capillary temperature 330°C, capillary voltage 47.5 V, tube lens voltage 115 V, vaporizer temperature 330°C.

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Statistical Analysis. The data were subjected to analysis of variance (One-Way ANOVA). The SPSS 17.0 statistical package was used for the evaluation of statistical significance of the differences between mean values by Tukey's and Duncan test. $P<0.05$wasconsidered statistically significant for the results.

Results and Discussion

Contribution of curcumin to the acrylamide formation in model systems

Acrylamide formation is the result of thermally induced conversion of ASN in the presence of carbonyls at elevated temperatures. In this study, potential contribution of CUR on acrylamide formation was investigated in different model systems.

In the absence of a carbonyl compound, 0.01 mmol of ASN heated for 30 min at 180°C generated 6.46×10^{-6}mmol of acrylamide (**Fig. 1a**). Granvogl et al. (2004) have previously reported that ASN alone is capable of forming acrylamide in limited amounts. However, it is well known that during the development of Maillard reaction carbonyl compounds promote free amino acids decarboxylation (through Strecker degradation) thus increasing acrylamide formation. The rate of acrylamide formation increased significantly in the presence of equimolar amounts of FRU and CUR in the model reaction system. The initial rate of acrylamide formation was approximately 20 times faster in the model system of ASN-FRU than that of ASN-CUR. This indicated that FRU was more reactive than curcumin from the viewpoint of acrylamide formation. In the ASN-FRU model system, acrylamide concentration reached to a maximum of 2.27×10^{-4}mmol within 10 min at 180^{o}C, followed by a rapid exponential decrease afterward. On the other hand, in the ASN-CUR model system acrylamide concentration increased linearly within 30 min at 180^{o}C reaching to 6.28×10^{-5} mmol. This evidence suggests that in the first case the formed acrylamide is eliminated by further reaction with proteins and other components of the system (Hidalgo et al. 2010). A similar behavior was found during coffee roasting where dark roasted coffee has less acrylamide than light roasted ones (Senyuva and Gökmen, 2005). When curcumin instead of FRU is present, the lower reactivity allowed ASN to sustain the formation of acrylamide over a long time (up to the 30 min monitored). To confirm this hypothesis, the concentration of reactants remaining in the model systems was also monitored during the reaction. In the ASN-FRU model system, the concentrations of ASN and FRU rapidly decreased, and only traces of these reactants were detected after 5 min of reaction at 180^{o}C. The rates of ASN and CUR disappearance were significantly slower in the ASN-CUR model system. Approximately 30% of ASN and curcumin still remained in the reaction mixture after 30 min of heating at 180^{o}C (**Fig 1b**). Therefore, it was considered that CUR could potentially contribute to acrylamide formation under long term heating conditions as long as asparagine is present in the medium. While there was a significant browning development in ASN-

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FRU model system, no color change was observed in ASN-CUR model system.

Competition between CUR and FRU in a ternary (ASN-FRU-CUR) system was also investigated. The amount of acrylamide formed in the model system of ASN-FRU-CUR was slightly higher than that of ASN-FRU during heating ($p<0.05$), because of the higher concentrations of carbonyl compounds initially available in the ternary system (**Fig 2**). The kinetic trends were similar in both model systems. This was a clear evidence of FRU reacting with ASN predominantly in comparison to CUR.

Mechanism of acrylamide formation in ASN-CUR model system

The results of present study propose that the reaction between CUR and ASN at elevated temperatures should proceed according to the reaction scheme shown in **Fig 3**. During heating the ASN-CUR model system, it was expected that carbonyl group of CUR would react with α-amino group of ASN forming corresponding Schiff base. The reaction products formed in the model systems during heating at 180^{o}C were analyzed by high resolution mass spectrometry by performing full scan, *m/z* ranged between 50 and 500. The analytical conditions applied here successfully resolved the peaks of intermediates formed in the model system during heating. To confirm the molecular structures of the intermediates and products, their observed masses were compared with corresponding theoretical masses. The results of high resolution mass spectrometry analyses indicated the presence of parent $[M+H]^+$ ion having m/z of 483.1762 (Δ=0.04 ppm) confirming the formation of Schiff base in the reaction mixture during heating (**Fig 4**). In the same way, the presence of decarboxylated Schiff base was also confirmed by detecting the ion having m/z of 439.1864 (Δ=0.02 ppm).

Formation of acrylamide in the ASN-CUR model system was confirmed by detecting $[M+H]^+$ ion having m/z of 72.0445 (Δ=2.77 ppm). It is known that decarboxylation of Schiff base can produce acrylamide through different paths. It has been previously reported that decarboxylated Schiff base may directly form acrylamide via β-elimination (Stadler et al. 2002; Yaylayan et al. 2005). On the other hand, acrylamide may be formed via Hofmann elimination (Locas and Yaylayan, 2008). Also decarboxylated Schiff base may be a direct precursor of acrylamide (Yaylayan et al. 2003) or hydrolyze to 3-APA that is capable of yielding acrylamide through deamination (Zyzak et al. 2003). These descriptions are consistent with the reaction scheme of CUR with ASN shown in Fig 3.

In the ASN-CUR model system, formation of 3-APA during heating was confirmed by detecting $[M+H]^+$ ion having m/z of 89.0709 (Δ=0.11 ppm). The amount of 3-APA formed in the ASN-CUR model system during heating at 180^{o}C was found to be 1.67×10^{-7}mmol, 2.42×10^{-7}mmol, and 1.45×10^{-7}mmol after 10 min, 20 min, and 30 min, respectively. Prolonging the heating time from 20 min to 30 min at 180^{o}C decreased the amount of 3-APA while the amount of acrylamide increased.

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Temperature dependence of acrylamide formation in ASN-CUR model system

The reaction mixture composed of equimolar amounts of ASN and CUR was heated at 150, 180 and 200^{o}C for different times up to 60 min. As shown in the **Fig 5a** the rate of acrylamide formation increased as the temperature increased**.** Data also revealed that acrylamide formation in a temperature range of 150-200^{o}C under low moisture conditions obey the Arrhenius law (**Fig 5b)**. The activation energy was calculated to be 79.1 kJ/mole from the slope of the plot of *1/T* versus ln*k*. This result is in the order of magnitude of those found by several previous studies dealing with the activation energy of acrylamide formation in different model systems and foods. Granda and Moreira (2005) have reported the activation energy of 61 kJ/mole for acrylamide formation in fried potato slices. The activation energy ranged between 58 and 95 kJ/mole depending on the step in the reaction pathway of acrylamide formation (Knol et al., 2005).

Effect of Curcumin on Acrylamide Formation in the Crust Model

In order to determine the contribution of CUR on acrylamide formation in foods, a bread crust model system having 1.0% of curcumin and 1.0% of glucose were prepared by baking at 180˚C for 10, 20, and 30 min. This bread crust model system proved to be effective in simulating the amount and the mechanisms of acrylamide formation in bread (Gokmen et al. 2009). As shown in **Fig 6**, the control sample without any carbonyl compound contained 346, 1378, and 2250 ng/g of acrylamide after 10, 20 and 30 min of baking at 180˚C, respectively. Acrylamide content of crust models containing 1.0% of GLC was significantly higher than that of samples having 1.0% of CUR just after 10 min of baking at 180˚C ($p<0.05$). On the other hand, acrylamide concentration of crust models containing 1.0% of CUR was found to be slightly higher than that of containing 1.0% of GLC when baking time was extended ($p>0.05$). GLC consumed free ASN rapidly due to its high reactivity. Acrylamide content of crust models reached maximum levels in a short time, while acrylamide formation proceeded at a slower rate in crust models containing CUR. The effect of CUR on acrylamide formation in crust models composed of 1.0% CUR and 1.0% GLC was also investigated. From the viewpoint of acrylamide formation, GLC and CUR (or any other carbonyls) competed for reaction towards ASN. Since the medium has a fixed amount of ASN, the resulting acrylamide concentration would be dependent on the rate and yield of individual reactions leading to acrylamide. Initially, the addition of CUR beside GLC reduced the amount of acrylamide generated, but it is clearly observed that CUR beside GLC in crust models promoted acrylamide formation with further heating (**Fig 6**).

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Conclusion

The literature reported contradictory evidence on the effect of antioxidants on the formation of acrylamide: in some conditions they work as mitigatory agents in others they promoted its formation (Capuano and Fogliano, 2011). Data of this work highlighted the possibility that antioxidants having a carbonyl group (as it is in curcumin, but a carbonyl moiety is formed upon oxidation of many phenolic compounds), can react directly with ASN leading to the formation of acrylamide. The reactivity of the carbonyl group present in curcumin is lower than that of reducing carbohydrates, however the peculiar kinetic of the ASN conversion into acrylamide catalyzed by curcumin can be relevant when it is added to the recipes for the preparation of functional foods or seasoned bread. Particular attention should be paid when the free sugar concentration is low and the ASN concentration is relatively high. In fact, in this case, the addition of antioxidants can probably increase the formation of acrylamide upon long term heating.

Conflict of Interest

The authors declare that they have no conflict of interest.

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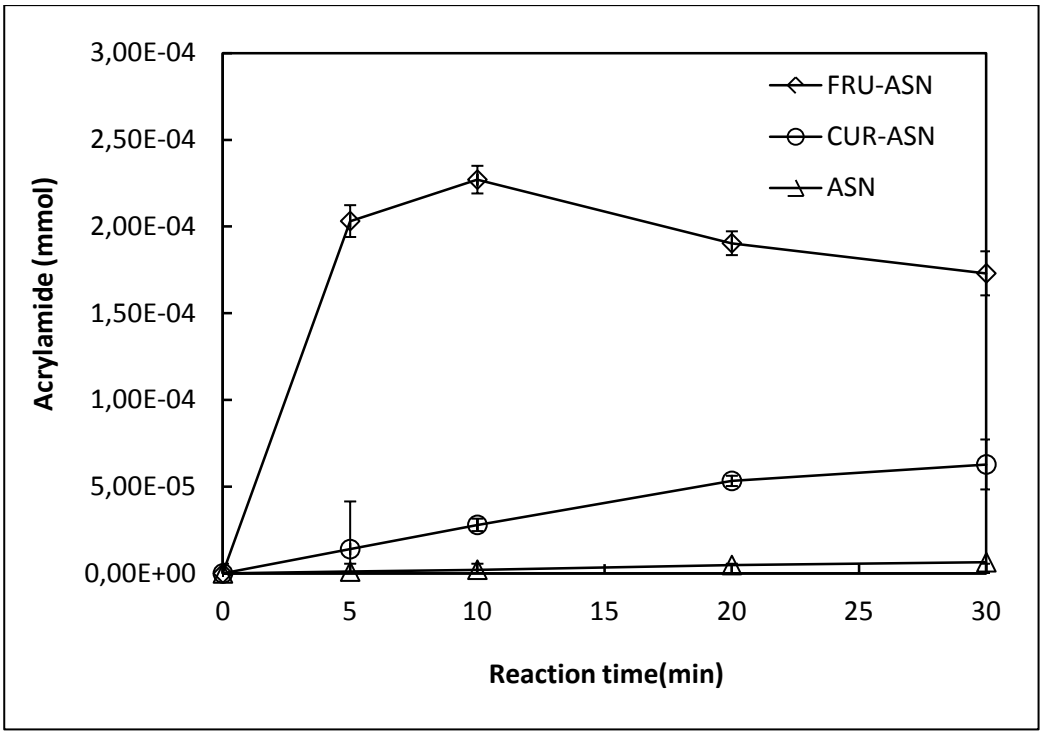

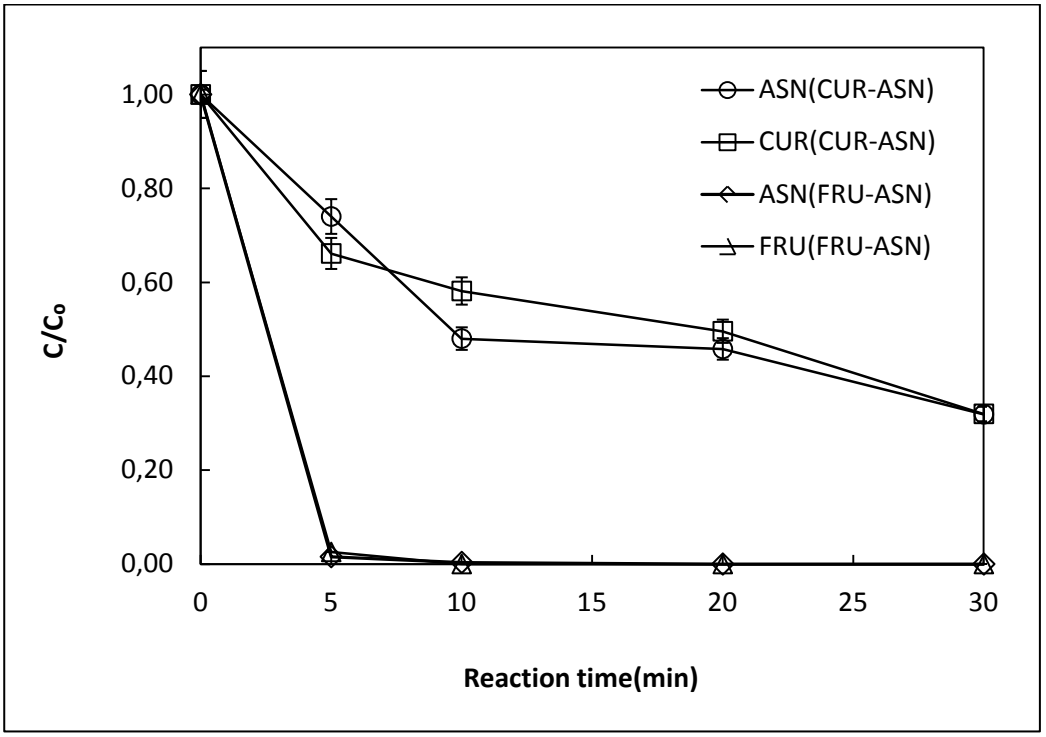

Figure 1 (a) Formation of acrylamide in ASN, CUR-ASN and FRU-ASN model systems during heating at 180^{o}C. **(b)** Change of reactant concentrations with time in during heating at 180^{o}C

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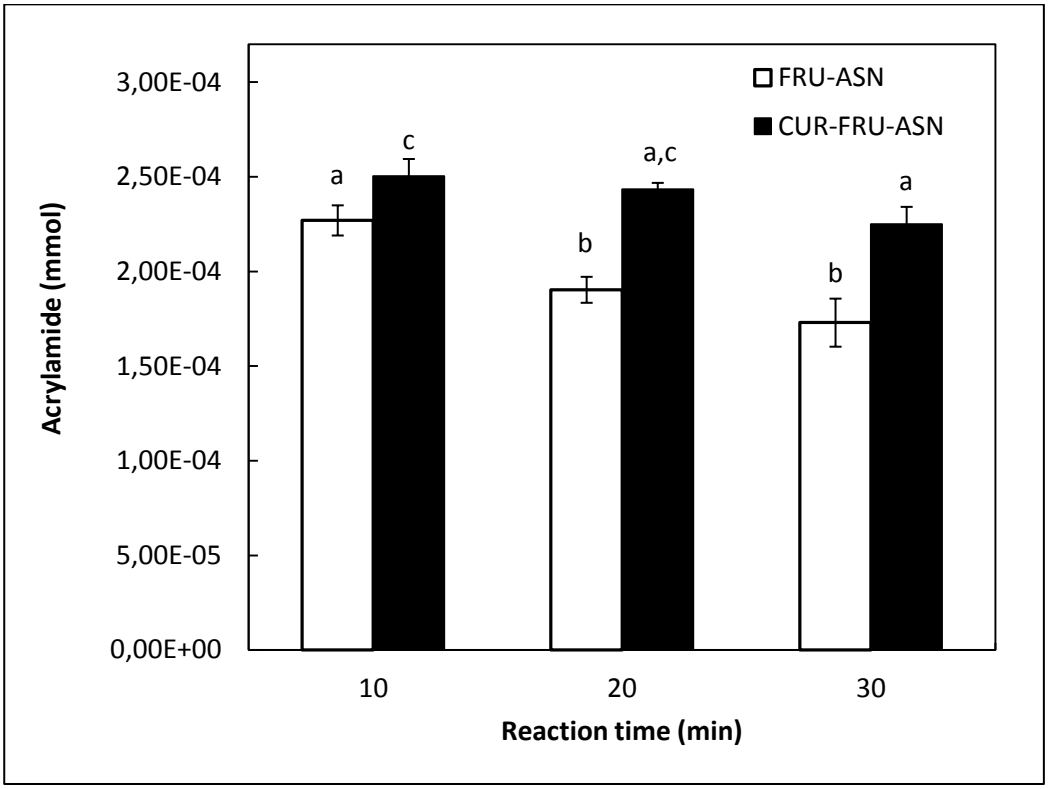

Figure 2. Formation of acrylamide in FRU-ASN and CUR-FRU-ASN model systems during heating at 180^{o}C

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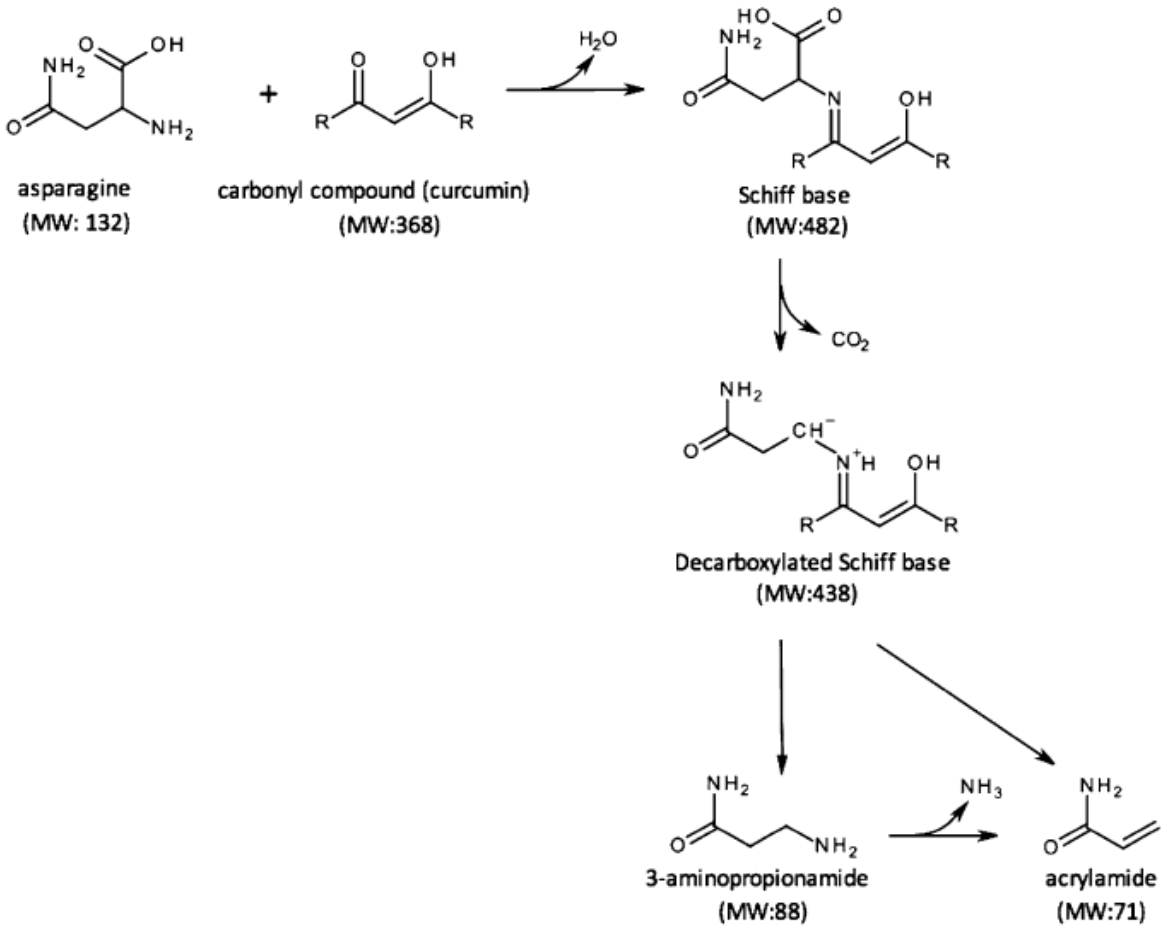

Figure 3. Proposed mechanism for the contribution of CUR on acrylamide formation from ASN

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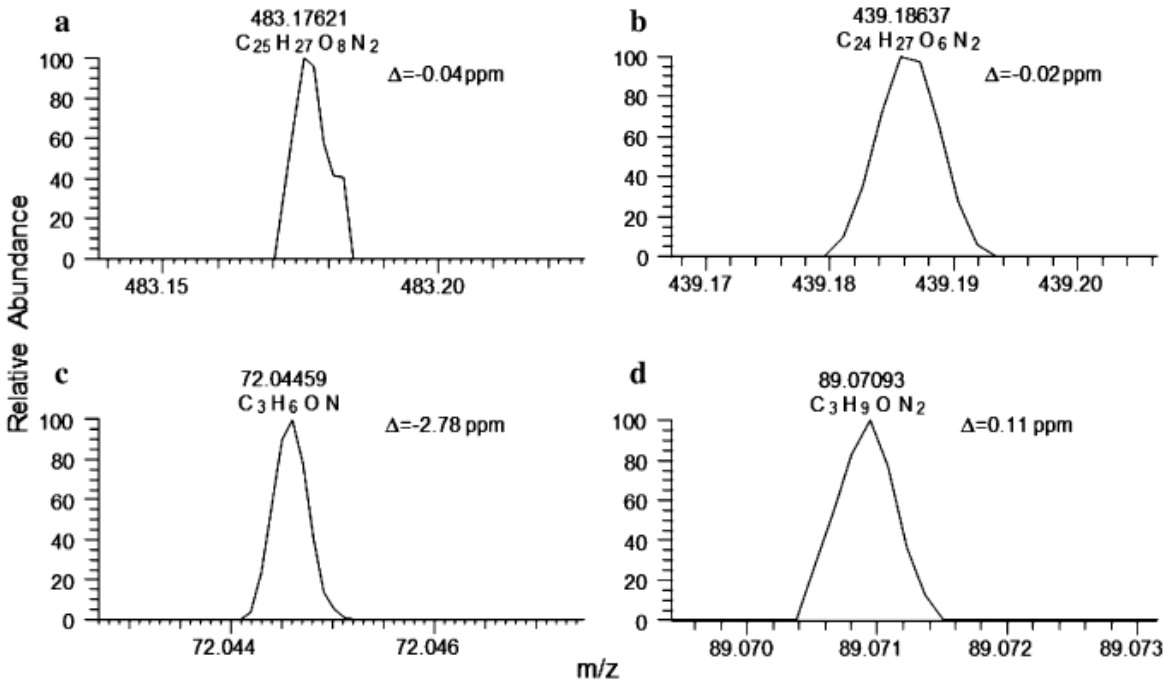

Figure 4. High resolution mass spectrometry confirmation of the reaction intermediates and products in ASN-CUR model system heated at 180°C. (a) Schiff base, (b) decarboxylated Schiff base, (c) acrylamide, (d) 3-aminopropionamide

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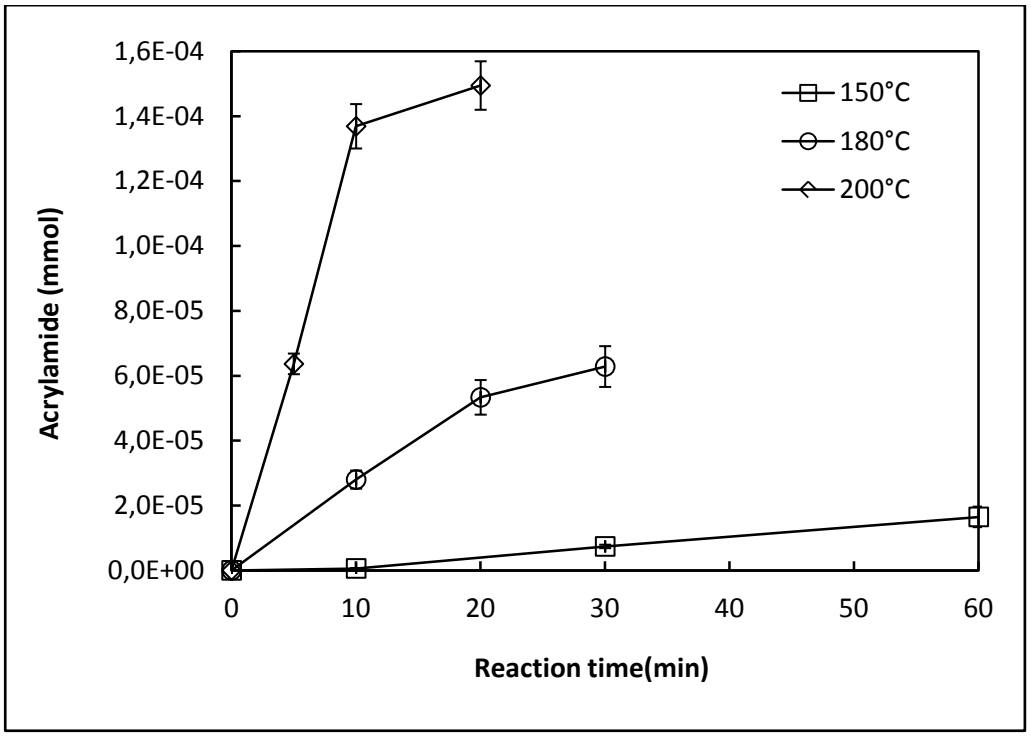

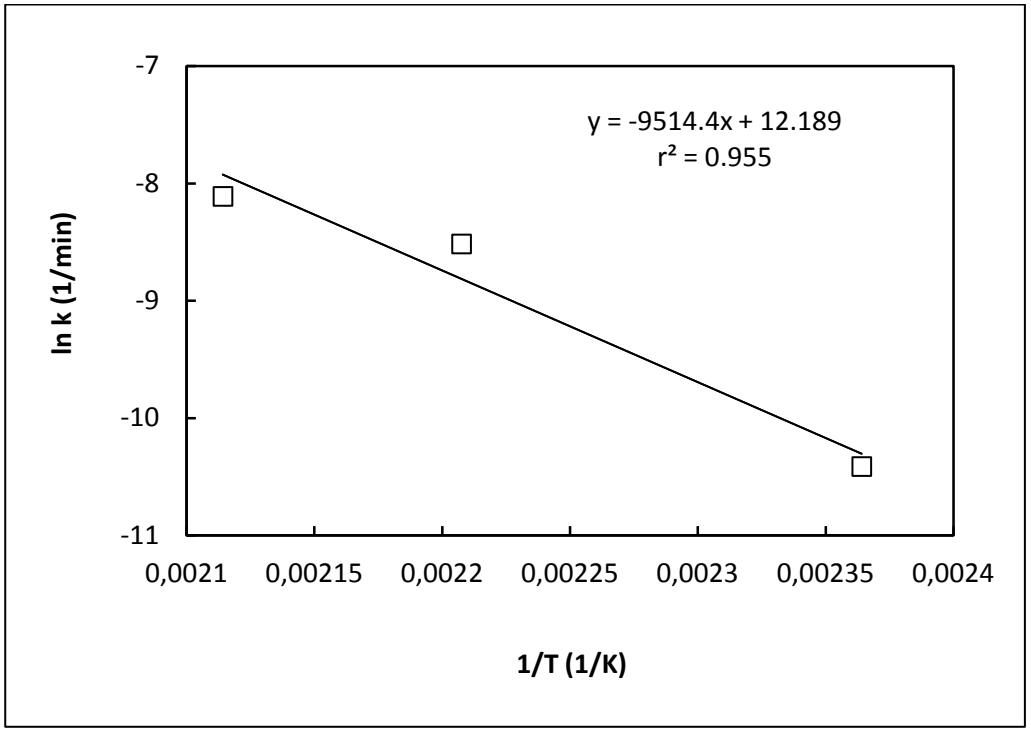

Figure 5 (a) Effect of temperature on acrylamide formation in CUR-ASN model system during heating up to 60 min, **(b)** Arrhenius plot of acrylamide formation in CUR-ASN model system

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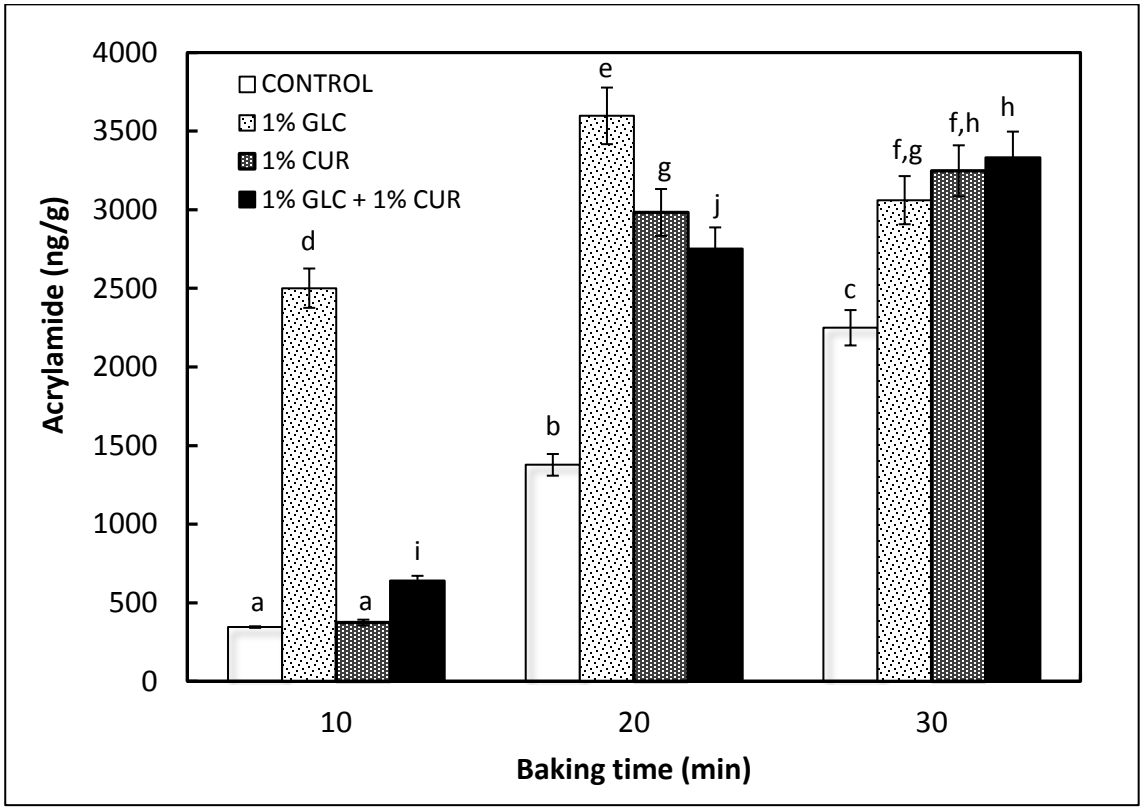

Figure 6. Amounts of acrylamide formed in control, 1% CUR, 1% GLC, 1% CUR+1% GLC crust models during heating at 180°C for different times

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A.4

Chemical profile and sensory properties of different foods cooked by a new radiofrequency oven

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Keywords

Radiofrequency heating, Vitamins, glucosinolates, acrylamide, sensory profile

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Abstract

Radio frequency (RF) heating has been used for numerous applications in the food industry such as baking, thawing or pasteurization. It reduces cooking time, and it helps to retain acceptable food colour and texture. In this paper, chemical and sensory data obtained from broccoli, potatoes, salmon and cocoa cakes cooked using an innovative RF oven were reported. The oven has an algorithm able to monitor the energy feedback from the cavity and to adjust the energy output accordingly. The different foods were cooked to the same end point and the concentration of phytochemicals, vitamins and acrylamide were assessed. Results demonstrated that RF oven preserved ascorbic acid and increased glucosinolates concentration in broccoli and it decreased the formation of acrylamide in roasted potatoes more than 50%. The total amount of vitamins B was 30 and 50% higher in RF cooked salmon than conventionally cooked salmon prepared at 55 and 75°C, respectively.

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1. INTRODUCTION

Since the discovery of the fire humans transferred heat to food materials to improve digestibility, to reduce possible pathogen contaminations, to increase shelf life and finally to obtain better sensory properties (Van Boekel *et al.*, 2010)

Conventional methods applied to transfer heat to food include hot air or water, steam, grilling by direct conduction, frying with oils and radiant heating. In all these heating methods the food is heated externally through conduction, convection or radiation which slowly penetrates to the food core. In all cases the heat transfer is not closely controlled and it is dependent on the modifications of the intrinsic characteristics of the food material, which are in turn continuously modified by the heating process (Marra, Zhang & Lyng, 2009).

In general, the bigger the size of the food pieces, the longer the cooking time required to ensure that the core of the products is ready. However, because of the dependency on heat transfer from the outside surface of the product to its interior, the surface may be overcooked, potentially reducing the product quality and its nutritional value (Friedman, 2003).

The food heating process is completely different when the energy transfer was performed using radio waves. Radio frequency (RF) heating is achieved through a combination of ionic displacement and dipole rotation. At higher frequencies (1000-30000 MHz), which includes the range of home microwave ovens (MW), dipole rotations are the major contributor to the heating mechanism, whereas at the RF lower frequencies (10-1000 MHz), ionic displacement became more important (Jones, Lelyveld, Mavrofidis, Kingman & Miles, 2003; Buffler 1993; Farag, Lyng, Morgan & Cronin, 2009).

Radio frequencies heated food more uniformly and thus reduced heating time. The heat is generated within the product due to molecular friction resulting from oscillating molecules and ions caused by the applied alternating electric field. Since the energy and heat are absorbed directly by the food, the RF cooking not only saved time but also energy (Zhao, Flugstad, Kolbe, Park, & Wells, 2000). Unlike conventional air systems, microwave (MW) and radio frequency (RF) systems generated heat within the product, which is also known as “volumetric heating” (Jamieson & Williamson, 1999; Rowley 2001). Ionic depolarization was the dominant heating mechanism at lower frequency while dipole rotation became very important at frequencies relevant to MW heating depending upon the moisture and salt content of the product (Tang, Wang & Chan 2005). During RF heating, electromagnetic power penetrated deeper into samples without surface over heating or hot spots

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developing which were more likely to occur with MW heating (Marra, De Bonis & Ruocco, 2010; Marra *et al.*, 2009).

RF cooking has already been used for numerous applications in the food industry since the 1940s such as baking, heating, thawing or pasteurization. It has been demonstrated that RF cooking reduced cooking time, lower juice losses, kept acceptable colour and texture and increased shelf lifeand it is particularly suitable for meat defrosting (Brunton, Lyng, Li, Cronin, Morgan, & McKenna, 2005; Guo, Piyasena, Mittal & Gong, 2000; Farag, Marra, Lyng, Morgan & Cronin 2010). The major limitation of the available RF technologies was the low number of efficient electromagnetic modes achievable in a tight band of frequencies resulting in a non uniform heating profilewith hot and cold spots within the cooked foods (Marra *et al.* 2009, Piyasena, Dussault, Koutchma, Ramaswamy, & Awuah, 2003).
In this paper data obtained cooking different foods by an RF cooking system device named IBEX (ITW, Chicago, IL, USA) that integrates a proprietary RF technology (GOJI Limited, Bermuda) into a convection cooking system are shown. The RF system is able to control the application of energy to the food by using a closed feedback loop. The aim of this work was to compare the nutritional profile and the concentration of a potentially harmful compound generated by thermal treatment. The experiments were run on broccoli, potatoes, salmon and cocoa cake which were cooked by the RF oven and by using conventional cooking (conventional, steam or microwave ovens). Beside the chemical composition also the sensory differences between samples cooked by RF or conventional procedure were investigated.

2. MATERIALS AND METHODS

2.1 Materials

Broccoli, potatoes, sweet potatoes, and salmon were purchased from local market. Ready to cook cake (Cameo, Italy) was used for sensory experiments. The conventional oven was a ventilated oven SMEG ALFA43 (SMEG SPA, Italy); the MW oven was an LG MH 6889 model (ALK LG, Seoul, South Korea). The steam oven was a CE20FD model (Hobart, IL, USA).
The IBEX RF oven manufactured by ITW (Chicago, IL, USA) was equipped with a computer algorithm that monitors feedback from the cavity and adjusts the energy transfer based on the feedback resulting in a uniform and controlled heating pattern. The total energy that the device will transmit depends on the state and matter of the cooked material. Consequently, the system applies the exact needed energy to the material without wasting energy on heating the cavity or over heating the material.

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The system used a computer algorithm that monitors Electro-magnetic (EM) feedback from the cavity as every specific material in a specific cavity has a unique EM absorption signature.
The oven has a power of 1000W, transmitting selectively between 800-1000 MHz. The amount of RF energy that the device will transmit is calculated based on the EM feedback. The EM feedback can be measured at different interval times during the processing and the transmitted RF energy is adapted as a function of the amount of energy absorbed by a particular material. Consequently, the system applies the exact needed energy to the material without wasting energy on heating the cavity or over heating the material
The thermocouple used, for measure in the core of the product, was a thermometer of Tersid SRL (Milan, Italy) equipped with a thermocouple type K (Omega, CA, USA).
Solvents used for acrylamide HPLC analysis were purchased from Merck (Darmstadt, Germany). Acrylamide (>99.5% purity), Potassium ferrocyanide (Carrez I) and zinc acetate (Carrez II) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acrylamide, [2,3,3-d_3]-acrylamide (isotopic purity 99%) was from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile, methanol, hexane and water were all of HPLC grade and were purchased from Merck (Darmstadt, Germany).

2.2 Food sample preparation:

Fresh broccoli samples were purchased from the local market, cleaned, cut and inserted in a plastic box before the cooking procedure. Potatoes were peeled and cut in a cube with an height of 15mm. Round shape sweet potatoes (*Ipomoea batatas*) of about 100 g each were cooked as a whole without peeling. Fresh whole salmon was purchased at local market and it was sliced in rectangles of 5 x 3.5 x 3.5cm. A commercial preparation (Cameo, Italy) of cocoa ready to cook cake, containing 600 g of a liquid dough was used.

2.3 Cooking procedure

Two kilograms of broccoli were chopped taking uniform samples of the apical parts and three homogeneous samples of 200 g were prepared. A set of preliminary experiments was performed by using the two cooking systems to find the conditions able to reach the same cooking end point
The RF samples were cooked in closed plastic box in the RF oven transferring 180 kJ without convectional heating in the oven (cold cavity), while the steam cooking was performed in a steam oven form for 8 minutes with an oven temperature of 100°C.
Potatoes cubes were cooked in two different way, in conventional oven and in RF combined oven.
The treatment were as follow: for the RF combined cooking oven temperature at 230°C cooking

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time 15min energy transferred 400 kJ , while the conventional oven (same oven without turn on the RF) condition were first cooking at 210°C for 41 min than temperature was lowered to 180°C for 11 minutes for a total cooking time of 52 min.

The cooking parameters of the sweet potatoes samples were as follow: for the RF combined cooking the oven temperature was set at 250°C the total cooking time was 15 min, the total energy transferred was 374 kJ; for the conventional cooking the oven temperature was 250°C the total cooking time was 30 min. After cooking, the crust was separated manually using a suitable cutter; then the crusts were freeze-dried and later analysed for acrylamide.

Five hundred grams of salmon cut in cubes of 3 cm edge were cooked in the following conditions: for the RF the oven temperature was set to 250°C and the energy was 25 kJ for 100 g of salmon, the time to reach the core temperature of 55°C was 4.5 min. For the conventional oven conditions the temperature was set at 220°C and 9 min were necessary to reach a core temperature of 55°C.

For the ready-to-cook cake the end point was determined by the complete absence of sticky material at the core of the product. Cooking parameters were as follow: for the RF combined cooking oven temperature of 220°C cooking time 8 min transferred energy was 230 kJ, while the conventional oven (the same oven without turn on the RF) condition were temperature of 170°C, cooking time 35 min.

2.4 Chemical determinations

2.4.1 Acrylamide

Finely ground sample (1 g) was weighed into a 10 mL centrifuge tube. The sample was spiked with 100 µL [$^{13}C_3$] - acrylamide (10 µg mL^{-1}) and 5 mL of Milli-Q water was added. Mixture was vortexed and kept for 5 min at room temperature, and homogenized for 1 min (Ultra Turrax, IKA, Mod-T10 basic, Bohn, Germany). Then, 500 µL Carrez I and 500 µL Carrez II solution were added, vortex and stand for 10 min. Tubes were centrifuged at 4000 rpm for 15 min at 4 ºC. The supernatant (1-2 mL) was clarified onto a pre-conditioned Oasis-HLB cartridge. Firsts drops were discharged and rest of the eluate was collected in amberlite vials for LC–MS-MS analysis.

LC–ESI–MS/MS analysis was performed using API 2000 triple quadrupole mass spectometer (Applied Biosystem, Sciex, CA, USA), with a Electro Spray Interface (ESI), coupled to HPLC binary micropumps (Perkin Elmer, Anhaim, CA, USA). Analytical separation was achieved with an Inertsil ODS-3 column (25×0.46cm, 5µm) (GLC-Sciences, Tokyo, Japan) using isocratic elution with a mobile phase of 0.2% formic acid in water at a flow rate of 0.8 mL min^{-1}. The quantification was carried out in MRM (Multiple Reaction Monitoring) mode at m/z ratios of 72.1 and 75.1 for acrylamide and [2,3,3-d_3]-acrylamide, respectively. Moreover, an m/z 55.1 and 44.0 and a m/z of 58.0 and 44.0 corresponding to specific molecular fragments of acrylamide and [2,3,3-d_3]-

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acrylamide respectively were monitored. The ions were obtained through fragmentation by specific collision energy of a selected ion precursor, applying a voltage of 4.5 kV.

A delay time of 3 minutes was selected to avoid the introduction of co extracted matrix components into the MS/MS instrument prior acrylamide elution. The needle and cone voltages were set at 3.0 kV and 100 V respectively. Nitrogen was used as nebulizer gas (12.0 Lh^{-1}) and the source temperature was set at 350ºC. Acrylamide was quantified using a linear calibration function that was established with standard solutions of acrylamide and [2,3,3-d_3]-acrylamide dissolved in Milli-Q water (25 to 1000 µg/L). Acrylamide contents in sample extracts were calculated from the calibration curve and intercept value, taking into account the recovery calculated by means of [2,3,3-d_3]-acrylamide curve. The limit of the quantitation was set at 20µg kg^{-1}. Precision (reproducibility) was lower than 12% and recovery between 80 and 90%.

2.4.2 Vitamins B and C

After the homogenization of the samples by mixing, 2 g of premix was weighed in a 100 ml volumetric amber flask, 40 ml of water and 4ml of 2M NaOH were added and the suspension was vigorously shaken, then 50 ml of 1M phosphate buffer (pH 5.5) were added in order to lower the pH of the final solution to 7.0. The suspension was made up to the mark with water and it was sonicated 10 min in an ultrasonic bath Bransonic 12 (Carouge, Geneva, Switzerland). Aliquot of the latter solution was removed for the quantification of vitamins B5 and B8 whereas; a dilution of 20 or 40-fold with water was used for the quantification of B vitamins. The solution was filtered through a 0.22 mm Millipore syringe Millex TMGP filter (Bedford, MA, USA) before the LC-analysis.

Reversed-phase chromatographic columns: Prodigy C18 (250mm×4.6 mm, 5µm particle size, Phenomenex, Europe, GmbH), were tested. An HPLC (LC10, Shimadzu, Tokyo, Japan) with DAD operating at two wavelengths: 210 nm for vitamins B5 and B12, and 275 nm for vitamins B1, C, PP, B6, B9 and B2. The mobile phase was made of: (A) TFA 0.025%, (B) acetonitrile. The elution gradient was: solvent (A) for 5 min, followed by a linear gradient to solvent (B)–solvent (A) (25:75, v/v) mixture during 6 min. Then a second linear gradient to solvent (B)–solvent (A) (40:60, v/v) during 8 min, this mixture being held for 1 min. Finally, the initial conditions were re-established in 1 min and held for 4 min.

2.4.3 Glucosinolates

GLS were extracted using a Advanced Microwave digestion system following the method of Omirou *et al.,* 2009 and the relative LC/MS/MS analyses were carried out following the procedure described by Barbieri, Pernice, Maggio, De Pascale and Fogliano (2008).

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2.5 Physical determinations

*2.5.1 Texture***.**

For this measurement was used an TAPlus Texture Analyser from Lloyd Instruments equipped with Volodkevitch Bite Set in order to imitate incisor teeth shearing through the food sample. The set comprises upper and lower 'teeth' which, during the test, are brought together until nearly touching. The sample is positioned on the lower 'tooth' and the result is measured as the peak force required to bite through the sample. The water content was measured following the official analytical methods (AOAC, 1995)

2.6 Sensory evaluation

Salmon fillet and ready to cook cake samples were prepared, identified by three random digit codes and served to the judges. Salmon fillet samples consisted of parallelepiped 50mm x 50mm x 15mm. The crust of the ready to cook cake samples was removed and 28mm diameter and 30mm height cylinders were prepared with a special metal cable cylinder.

Fifty-five untrained subjects participated in the test, to reflect consumers' perception. Paired comparison tests were performed in order to evaluate if differences in terms of specific sensory properties existed between the samples cooked in the conventional and RF mode. Significance for the differences was established at an alpha risk of 5%.

Sensory tests were carried out in the Sensory Science Laboratory of the Food Science Department at the University of Naples. The data were collected by means of "FIZZAcquisition" software (Biosystèmes, Couternon, France).

2.7 Statistical analysis

Data were analyzed by ANOVA and means were compared by Duncan's Multiple Range Test.
Three repetition for each samples was considered

3. RESULTS AND DISCUSSION

The cooking experiments were designed in order to have the same cooking point at the end of the treatment with the different cooking procedures. To achieve this objective different parameters were selected for the various foods. The same texture for broccoli and roasted potatoes, the same core temperature for salmon and the complete gelatinization of the dough for the cocoa cake. The

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experimental plan is summarized in **Table 1** indicating for each food the sensory, chemical or physical parameters that were investigated.

3.1 Broccoli

The broccoli samples were analyzed at the same consistency for the two different cooking methods, in **Table 2** the texture parameters of both flowers and rod are reported. Samples cooked in the RF oven for 6 min and those cooked in the steamed oven for 8 min have similar texture parameters. Therefore, according to the experimental design above described, these samples were considered for the comparison of nutritional data.

The concentrations of single and total glucosinolates are reported in **Table 3**: their amount was more than doubled in the sample prepared by the RF oven respect to the fresh broccoli. In agreement with literature data steam cooked samples has a glucosinolates concentration not significantly different respect to the raw samples (Miglio, Chiavaro, Visconti, Fogliano & Pellegrini, 2008). The main glucosinolates compounds, namely the glucobrassicin is responsible for this increase, however glucoraphanin is decreased by the RF treatment thus suggesting that different glucosinolates can have a different sensitivity to the cooking systems. It is well known that mild cooking condition can lead to a better extractability of the samples due to the softening of the vegetable matrix, however, it has been observed that prolonging the treatment glucosinolates degradation become the prevalent phenomenon (Miglio *et al.*, 2008). The results obtained by RF treatment is worth to notice: in fact, glucosinolates are known to be thermo-labile compounds that can be preserved only with steam cooking. The concentration of Vitamin C was also monitored in the samples and shown in **Table 3**. The value are not different among the treated samples and similar to those of raw broccoli. This result indicated that both are mild treatments, as it is known that also ascorbic acid is a very thermo-labile compounds. In the case of vitamin C concentration no variation of its concentration respect to the raw broccoli was detected. This is probably because it is a small very soluble molecule so the matrix softening do not significantly affect its extractability as it happened for the glucosinolates.

3.2 Potatoes and sweet potatoes

The concentration of acrylamide on the potato surface was measured to verify the influence of cooking procedure on the presence of this important food contaminant. Potatoes were selected because among the different foods they contain one of the highest concentration of the main acrylamide precursors, namely the free asparagine. There is a general EU recommendation to apply all possible measures to reduce the acrylamide presence in foods to decrease the risk associated to the daily intake of this potentially harmful toxicants (Capuano & Fogliano, 2011)

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Potatoes were cooked to reach the same consistency (i.e to the complete starch gelatinization at the core of the potato cubes) as shown in **Table 4**. The cooking time was 30% shorter using RF in combination with conventional heating than using conventional heating only. Acrylamide concentration, given on a dried crust basis, are reported in **Figure 1**. Data showed that conventionally cooked roasted potato cubes have an acrylamide concentration double than those cooked by RF oven. The absolute values of acrylamide recorded in these samples of roasted potatoes are similar to those reported in the literature for potato crisps and this can be explained by the fact that only the crust of the cubes was analysed (Gökmen and Şenyuva 2006; Morales, Capuano & Fogliano, 2009)

The lower concentration of acrylamide in the RF cooked samples can be explained by the shorter cooking time also leading to a less brown potato surface. In fact, it is well known that potato surface colour is correlated with acrylamide concentration (Gökmen, Morales, Ataç, Serpen & Arribas-Lorenzo, 2009)

A similar trend was also observed regarding the acrylamide concentration in sweet potato cubes: in this case the acrylamide concentration was 250% higher in the conventional cooked samples. Sweet potatoes represent the worst case for acrylamide formation: they have very high concentration of free carbohydrates which are usually the limiting factor for the acrylamide formation in potatoes where the asparagine content is quite high and similar to that of the conventional potatoes.
All in all, acrylamide results suggested that RF cooking can be proposed as an efficient mitigation strategy for acrylamide formation in potato samples.

3.3 Salmon

Salmon samples were cooked in the oven combining RF with conventional heating and in the conventional oven only until they reach two different core temperatures 55 ± 2 °C and 75 ± 2 °C, respectively. Using RF oven the time of cooking was about the half than that of the conventional oven (4,5 min vs 9,0 min, respectively).
In **Figure 2** the total concentrations, obtained as the sum of the single B vitamins measured in the salmon samples, are shown. Data showed that salmon cooked by RF cooking have a concentration of B Vitamins significantly higher than that found after conventional cooking. Interestingly, the total concentrations recorded for B Vitamins in cooked samples was even higher than the raw salmon. This was probably due to the better extractability, caused by the matrix softening, achieved from cooked samples.

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Sensory results obtained by paired comparison tests are summarized in **Table 5**. They showed that a significant majority of the judges (39 out of 55; $p<0.01$) found the RF cooked salmon as the more tender one, whereas no difference was found in terms of dryness/juiciness.

The moisture content of the salmon sample cooked up to 55°C in the RF oven was significantly higher than that of samples cooked in the conventional oven (66.3 vs 57.1%). However, this difference was not enough to be significantly perceived in thedryness/juiciness sensory evaluation by the judges. However, tenderness and juiciness are closely related, the more tender the meat is, the more quickly the juices are released by chewing and thus the juicier the meat appears (Vasanthi, Venkataramanujam & Dushyanthan, 2007). Tenderness is strongly affected by the moisture content: water can have a plasticizing/lubricant effect that increases the perception of tenderness (Mora, Curti, Vittadini & Barbanti, 2011). Mounting evidence showed that tenderness of meat is related to the cooking procedure (Powell, Dikeman & Hunt, 2000) and cooking temperature (Chiavaro, Rinaldi, Vittadini & Barbanti, 2009), while no data on the effects of cooking procedure and heating temperature on the tenderness of fish fillet are available.

3.4 Ready-to-cook cake

A commercial liquid dough developed for the preparation of ready-to-cook cocoa cake were used for this experiment comparing again the cooking performance achieved by combining conventional and RF treatment with that achieved by conventional oven only. The cooking time of the cake was much shorter using RF cooking (8 min vs 25 min) and the leavening process of the cake was more reproducible with a final height of the cake which was more uniform in the RF oven than in conventional oven.

Sensory results frompaired comparison tests are illustrated in **Table 5**. Thirty-six subjects identified the RF sample as the softer one, indicating that a significant difference in softness was perceived ($p<0.05$). No difference was found in terms of dryness for the cake, even though the 58% of the subjects indicated the conventionally cooked sample as the drier one. In this case, the moisture content of the RF cooked sample was slightly higher than that cooked in the conventional oven (15.0% vs 12.4%).

CONCLUSION

In conclusion, RF-heating proved to be a very efficient technique to cook broccoli while preserving ascorbic acid and increasing glucosinolates concentration better that the best cooking procedure reported thus far for preservation of vegetable nutritional quality (i.e. steaming).

Anese, Sovrano & Bortolomeazzi (2008), firstly reported that RF heating is a promising strategy to reduce acrylamide in bakery products particularly at low water activities. The data on the

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acrylamide concentration reported in this paper confirmed that RF cooking might have interesting applications in the mitigation of different thermal contaminants, probably not only acrylamide. As a matter of fact, the possibility to uniformly and rapidly heat the material made RF cooking a technique with potentially great advantages over the other heat transfer technologies as far as the mitigation on undesired thermally neo-formed compounds.

The RF oven used in this study combined RF energy and conventional heating methods using an algorithm that monitors a feedback, then adjusts the energy transmitted resulting in a controlled heating pattern. The total energy transmitted by this device depended upon the state and matter of the cooked material. The system uses an ensemble (array) of exciters (antennas) that illuminate the cavity with RF radiation, and a real-time controller that feeds these exciters (and modulates the excitation) under the supervision of computer algorithm. The algorithm monitors the Electro-magnetic (EM) feedback from the cavity, as every specific material in the cavity with a determined geometry has a unique EM absorption signature. The computer algorithm enable a controlled energy transfer, and hence a controlled uniform heating of the material placed in the cavity. Consequently, this combined oven applies the exact needed energy to the material without wasting energy on heating the cavity or over heating the material. When compared to a microwave, this particular RF oven successfully cooked a much wider array of products and it did this much more uniformly. When compared to a convection oven, the IBEX oven performs baking and cooking tasks with a much better uniformity, and in a significantly shorter time.

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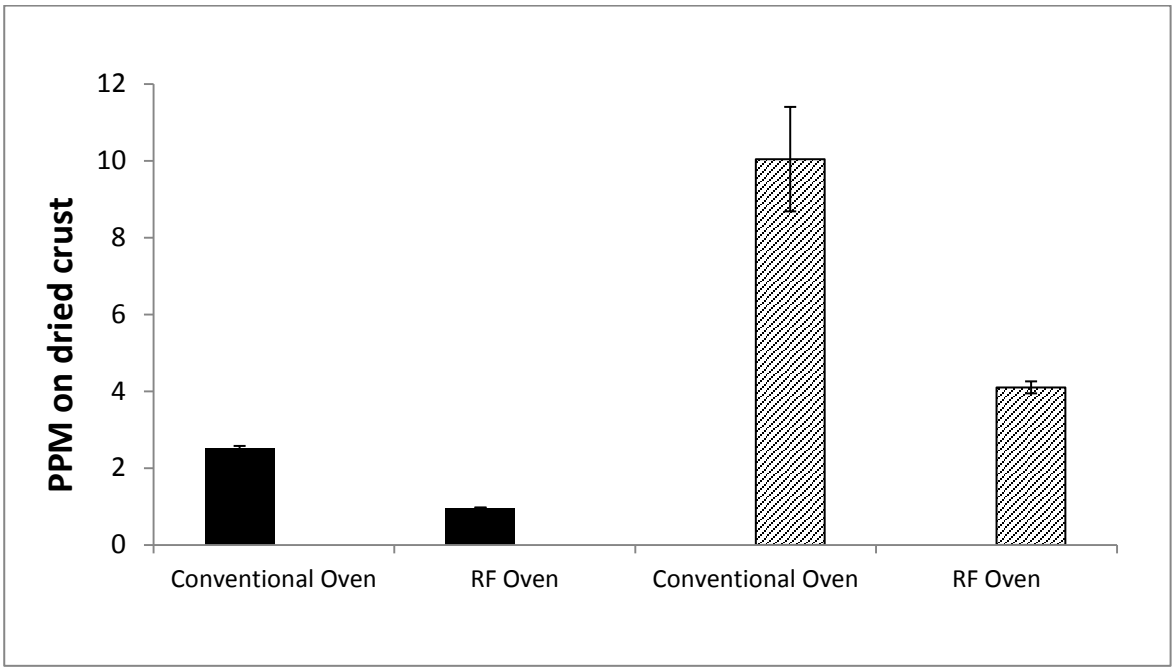

Figure 1 Acrylamide concentration in conventional (black bars) and sweet potatoes (gray bars).Samples were cooked by conventional and RF oven until they reached the same texture. The significant differences among cooking treatments were determined by Anova analysis and Duncan's multiple range test ($p \leq 0.05$).

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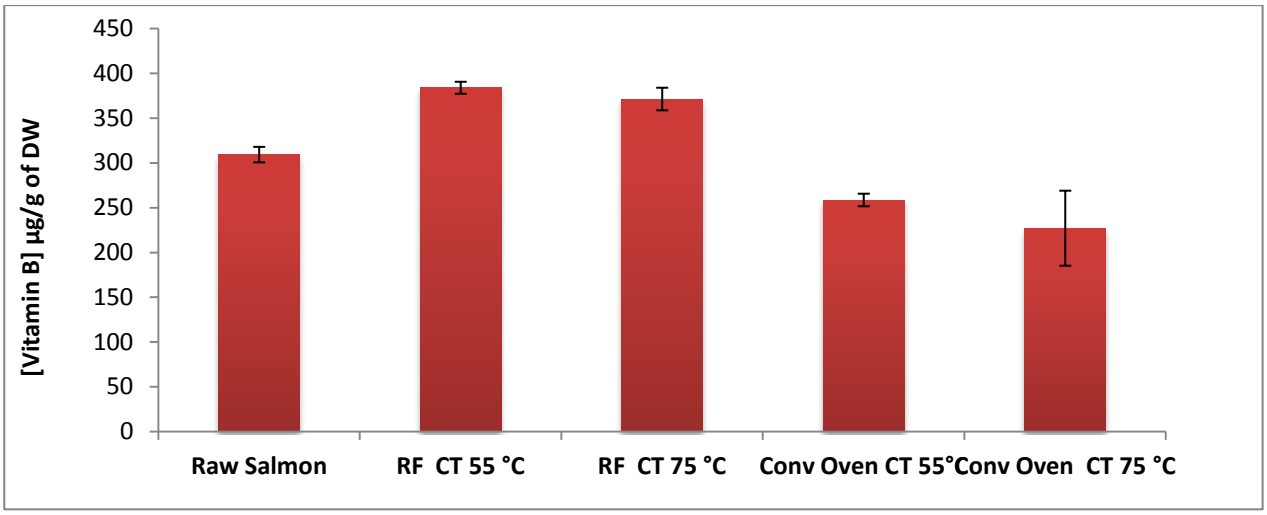

Figure 2: Total concentration of Vitamin of B groups (B1, B2, B3, B5, B6, B9, B12) in raw and cooked salmon prepared using RF and conventional ovens reaching core temperature of 55 ± 2 °C and 75 ± 2 °C.

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Table 1 Experimental plan for the four selected foods.

Food	**Cooking procedure**	**Goal to achieve with the cooking**	**Chemical, nutritional, safety and sensory parameters**
Broccoli	Steamingvs RF	Same texture	Glucosinolates, Vitamin C
Potato and sweet potatoes	Electric oven vs RF	Same texture	Acrylamide
Salmon	Electric oven vs RF	Core temperature 50°C	Juiciness,vitamins B
Ready-to-cook cake	Electric oven vs RF	Same water content	Softness

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Table 2: Broccoli texture parameters

	Raw broccoli				RF cooked broccoli				Steamcooked broccoli			
Samples	**Rod**	**SD**	**Flower**	**SD**	**Rod**	**SD**	**Flower**	**SD**	**Rod**	**SD**	**Flower**	**SD**
Texture (Newton)	53.9	4.0	44.9	3.3	17.4	6.8	19.3	4.8	15.6	1.2	14.6	0.8
Penetration distance (mm)	4.5	0.2	3.3	0.4	4.1	0.3	4.4	0.2	4.7	0.0	3.2	0.1
Diameter (mm)	10.9	0.9			12.1	1.6			11.2	0.2	12.6	2.4

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Table 3 Concentration of Vitamin C (mg per 100g fresh weight) , concentration odsingle and total glucosinolates (μmol*g^{-1} dry weight), in broccoli cooked with different methods. The significant differences among cooking treatments were determined by Anova analysis and Duncan's multiple range test ($p \leq 0.05$).

	Total GLS (μmol g^{-1} DW)		**4MetGLBr (μmolg^{-1} DW)**		**GLRaf(μmol g^{-1} DW)**		**GLEr (μmol g^{-1} DW)**		**GL Br (μmol g^{-1} DW)**		**VIT C (mg 100 g FW)**	
	Value	***SD***	***Value***	***SD***	***Value***	***SD***	***Value***	***SD***	***Value***	***SD***	***Value***	***SD***
Fresh broccoli	10.4[c]	0.7	1.1[b]	0.1	2.6[a]	0.3	0.3[a]	0.0	6.5[c]	0.2	72.6[a]	3.6
Steamed broccoli	13.1[b]	1.6	1.1[b]	0.0	2.5[a]	0.3	0.5[a]	0.1	9.0[b]	0.7	66.8[a]	5.0
RF cooked broccoli	23.7[a]	1.4	1.7[a]	0.1	1.2[b]	0.1	0.3[a]	0.0	20.4[a]	0.9	73.1[a]	8.0

4MetGLBr: 4-Methoxy Glucobrassicin, GLRaf: Glucoraphanin, GLEr: Glucoerucin, GLBr :Glucobrassicin)

Different letter along the column correspond to a statistically different value (a>b>c)

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Table 4 Texture parameters of cooked potato samples

		Average cube penetration (N) **	**SD**
RegularPotatoes	Raw Potato	54.8	4.12
	ConventionalOven	4.82	0.8
	RF Oven	6.06	1.01
		Average central slice (N) *	**SD**
SweetPotatoes	Raw Potato	121.88	3.66
	ConventionalOven	1.34	0.04
	RF Oven	1.24	0.04
(*) 10mm of penetration distance (**) 5mm of penetration distance			

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Table 5. One-side paired comparison tests' results on salmon fillet and ready to cook cocoa cake

Difference test	Sample	Oven type	Answers	Significance
Tender	**Salmon fillet**	RF Conventional	39 16	$p<0.01$
Dry		RF Conventional	25 30	n.s.
Soft	**Cocoa cake**	RF Conventional	36 19	$p<0.05$
Dry		RF Conventional	23 32	n.s.

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A.5

Quantitation of Acrylamide in Foods by High Resolution Mass Spectrometry

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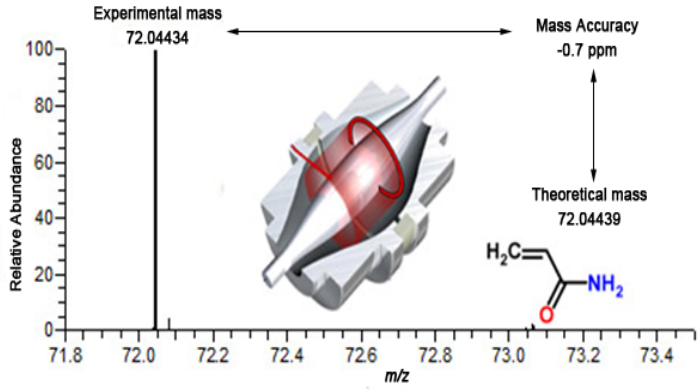

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Abstract

Acrylamide detection still represents one of the hottest topics in Food Chemistry. Solid phase clean up coupled to liquid chromatography separation and tandem mass spectrometry detection along with GC/MS detection are nowadays the golden standard procedure for acrylamide quantitation thanks to high reproducibility, good recovery and low relative standard deviation. High resolution mass spectrometry (HRMS) is particularly suitable for the detection of low molecular weight amides and it can provide some analytical advantages over the other MS. In this paper a liquid chromatography (LC) method for acrylamide determination using HRMS detection was developed and compared to LC coupled to tandem mass spectrometry. The procedure applied a simplified extraction, no clean up steps and a 4 minutes chromatography. It proved to be solid and robust with an acrylamide mass accuracy of -0.7 ppm, a limit of detection of 2.65 ppb and a limit of quantitation of 5 ppb. The method was tested on four acrylamide-containing foods: cookies, French fries, ground coffee and brewed coffee. Results were perfectly in line with those obtained by LC/MS/MS.

Keywords: Acrylamide, Orbitrap, High Resolution Mass Spectrometry, Maillard Reaction

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Introduction

Acrylamide has been a hot topic in Food Science and in particular in Maillard Chemistry[1, 2], since the first report highlighted its presence at high concentration in French fries, cereals and coffee[3]. On one hand, overlooking acrylamide toxicological and exposure outcomes[4-6], a huge variety of papers dealt with the chemical and analytical insights of acrylamide determination. The chemical aspects of acrylamide formation have been deepened in several pivotal papers: the determination of precursors[7-9], the detection of a key intermediate[10], the mechanism in heated food[11], the kinetic modeling of formation[12]. On the other hand, in the last decade several methods for its quantitative determination have been reviewed[13, 14]. Generally, acrylamide analysis can be characterized by three steps: sample preparation including extraction, spiking with labeled internal standard with derivatization or not[15], clean up, chromatographic separation and detection. As regards the first step several techniques have been developed providing satisfactory results: pressurized liquid extraction (PLE) cation-exchange cartridges, hydrophilic-lipophilic balanced cartridges, C-18 cartridges[16-19]. The chromatographic separations should take into consideration several drawbacks, among them the acrylamide high polarity along with the poor retention, the matrix effect and the solvent interferences[20-22] that can be ruled out through derivatization procedures with mercaptobenzoic acid or through the use of the "bromination" method[23-25]. For the detection step the analytical techniques of choice were the tandem mass spectrometry (MS/MS) acquisition performed by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) choosing the characteristic transitions *m/z* 72→55, 72→54 and 72→27[26-28], Fourier transform infrared (FT-IR)[8] analysis or by time of flight mass spectrometry (TOF/MS)[29], either or by fluorescence method[30]. In order to obviate the matrix effect and the poor retention of acrylamide and in addition to the above mentioned analytical methods, many official institutions standardized and published their full validated methods for the determination of acrylamide, most of which focused on LC/MS/MS analysis and the isotope dilution with ^{13}C or ^{2}H (Swiss Federal Office of Public Health; US Food and Drug Administration[28]).

In this paper the performances of ultra high pressure liquid chromatography coupled to Orbitrap High Resolution Mass Spectrometry (U-HPLC/HRMS) have been evaluated and accurately compared to the HPLC tandem mass spectrometry method (LC/MS/MS) in order to build a fast and sensitive procedure which can avoid the clean-up step as well as obtaining a high mass accuracy .

Materials and methods

Chemicals

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Methanol and water for U-HPLC/HRMS and LC/MS/MS determination were obtained from Merck (Darmstadt, Germany). Formic acid (98%), acrylamide, [2,3,3-d_3]-acrylamide and Orbitrap calibration solution standards were purchased from Sigma (St. Louis, MO). All of the samples were filtered through 25 mm diameter and 0.22 µm pore size nylon filter using a 2.5 mL syringe (BD, Franklin Lakes, NJ) equipped with a PTFE adapter (Phenomenex, Torrance, CA). Carrez reagent potassium salt and Carrez reagent zinc salt were purchased from Carlo Erba (Milano, Italy).

High Resolution Mass Spectrometry (HRMS)

Acrylamide separation was performed on an U-HPLC Accela system 1250 (Thermo Fisher Scientific, San Jose, CA) consisting of a degasser, a quaternary pump, a thermostated autosampler and a column oven. Mobile phase A was formic acid 0.1% and mobile phase B was 0.1 % formic acid in methanol. Four chromatographic columns were tested: Synergi Hydro (150 x 2.0 mm, 4.0 µm) Kinetex PFP (50 x 2.0 mm, 2.6 µm), Luna HILIC (150 x 2.0 mm, 3.0 µm), Kinetex C18 (100 x 3.0 mm, 2.6 µm) all from Phenomenex (Torrance, CA). In all cases the following binary gradient (min)/(% B): (0/5), (2/5), (4/80), (5/80), (6/5), (7/5) was used and the flow rate was 0.2 mL/min, except for C18 column where the flow rate was 0.3 mL/min and for HILIC column where the mobile phases consisted in A 0.1% formic acid in acetonitrile and B 0.1% formic acid in water. Volumes of 10 µL were injected using the thermostated autosampler at 18° C. The separation temperature was set at 30°C and the autosampler needle was rinsed with 500 µL of a mixture of methanol/water 50/50 before each injection.

To set up the optimal condition a water solution of acrylamide (concentration 250 µg/mL) at a flow rate of 5 µL/min was pumped using a 500 µL syringe pump directly into the LC stream entering in the HRMS (6.25 µg/mL final concentration, according to the chromatographic flow rate that was 0.195 ml/min). The U-HPLC was coupled to an Exactive Orbitrap MS (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray interface operating in the positive mode and scanning the ions in the *m/z* range of 50–400. The resolving power was set to 50,000 full width at half maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s. The automatic gain control was used to fill the C-trap and gain accuracy in mass measurements (ultimate mass accuracy mode, $5x10^5$ ions); maximum injection time was 50 ms. The interface parameters were as follows: the spray voltage 4.5 kV, the capillary voltage 42.5 V, the skimmer voltage 14 V, the capillary temperature 300 °C, the heater temperature 250 °C and a sheath gas flow 40 and auxiliary gas flow 6 (arbitrary units).

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Before starting the acrylamide determination the instrument was externally calibrated by infusion with a calibration solution that consisted in caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v). Several reference masses (lock masses) were tested for the mass analyzer recalibration: methanol ($[M_2+H]^+$, exact mass: 65.05971); methanol/water ($[A_2B_2+H]^+$, exact mass: 101.08084); sodium adduct of methanol/water ($[A_2B_2+Na]^+$, exact mass: 123.06278); [2,3,3-d_3]-acrylamide ($[M+H]^+$,exact mass: 75.06322) and diisooctyl phthalate ($[M+H]^+$, exact mass: 391.28429)[31]. In order to optimize the HRMS conditions and the mass accuracy, 200 µL of the above mentioned calibration solution were spiked with 10 µg of [2,3,3-d_3]-acrylamide and the scanning window was set in the range 70 – 1400 *m/z* only for the initial calibration procedure.

Preparation of standard solutions

A stock solution of acrylamide was prepared dissolving 10 mg of standard in 1 mL of water. This solution was diluted and stored at -20°C until the use. A calibration curve was built in the range 5 - 500 ng/mL according to the limit of detection (LoD) and to the limit of quantitation (LoQ). Three replicates of the solutions 0.5 ng/mL were injected into the U-HPLC/HRMS system to verify the lowest concentration for which the signal to noise ratio was higher than three. Concentration lower than 1 ng/mL resulted in no signal. The LoQ was 5 ng/mL for the standard solution and the r^2 value was always higher than 0.99 in the above mentioned range. Reproducibility of the method was evaluated through the intra-day and inter-day assay. The slope among the three subsequent calibration curve showed an RSD% lower than 7%. The same calibration points used for the above mentioned curve were spiked with 1.25 µg of [2,3,3-d_3]-acrylamide in order to evaluate the potential differences in presence or in absence of the internal labeled standard.

Sample preparation

Cookies. Model cookies (height 3 mm diameter 30 mm) were prepared according to AACC method 10-54 (AACC, 2000) as previously described[32]. Cookies were baked at 200 °C for 13 min in a forced-air circulation oven (Memmert, Schwabach, Germany).

French fries. Ten pieces (5 cm x 0.7 cm x 0.7 cm, total amount 50 g) were fried in one liter of olive oil for five and seven minutes. At the end of the cooking process the French fries were freeze dried and stored until the acrylamide extraction.

Brewed coffee and ground coffee. Ground coffee was purchased in a local market; an aliquot was directly analyzed without further preparation, instead 15 g were brewed with 100 mL of water (100

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°C for 15 min) under continuous stirring. Solid particles were removed through a paper filter and 50 ml were stored at -20 °C until the analysis.

Acrylamide extraction

French fries and cookies were ground in a knife mill Grindomix 200 (Retsch, Haan, Germany) and 100 mg were weighed in a volumetric flask; along with 4.8 mL of deionized water, 100 µL Carrez reagent potassium salt and 100 µL Carrez reagent zinc salt were added to each sample. The same protocol was followed for ground coffee. For coffee liquid samples 500 µL were diluted in 1 mL of deionized water. Each replicate of the different samples was prepared in two different ways: one was spiked with 125 µg of [2,3,3-d_3]-acrylamide while the other without the internal standard. All samples were vortexed for 10 min at 800 rpm at room temperature and after centrifugation (2500 x g, 4 °C, 10 min), 1 mL of the aqueous layer was accurately filtered and collected into glass vial.

Tandem mass spectrometry (MS/MS)

In order to validate the HRMS method all the above mentioned samples were analyzed through LC/MS/MS according to Gökmen et al[16]. Two grams of the freeze dried French fries, cookies or coffee powder were weighed; 8.9 mL of deionized water was added in a 15 mL centrifuge tube along with 500 µL of Carrez reagent potassium salt, 500 µL of Carrez reagent zinc salt and 100 µL of internal standard [2,3,3-*d3*]-acrylamide (final concentration 90 ng/mL). For brewed coffee one mL was mixed along with 8.9 mL of water, 500 µL of both Carrez reagents and 100 µL of internal standard. The tubes were shaken vigorously for 1 min and the resulting mixture was centrifuged at 2500 x g for 10 min at 4 °C. Then supernatant was collected in a 20 mL volumetric flask, and two further extractions were performed using 5 mL of deionized water, except for brewed coffee that was filtered and injected. Finally, the pellets were discarded, and the supernatants were filtered through a 0.45 µm nylon filter. One milliliter of supernatants was collected and passed through an Oasis HLB cartridge (Waters, Milford, MA) previously activated with 1 mL of methanol and 1 mL of deionized water; 20 µL of the final solution was injected onto the LC column for quantitation by MS/MS. Identification and quantitative determination of acrylamide and [2,3,3-*d3*]-acrylamide were carried out using an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA) coupled to an ion spray interface, equipped with an HPLC binary micropump series 200 (Perkin-Elmer, Waltham MA). Chromatographic separation of acrylamide and [2,3,3-*d3*]-acrylamide was achieved through an Inertsil column, 250 × 4.6 mm, 5 µm (GLSciences, Torrance, CA), the mobile phases were A: 0.1% formic acid and B: 0.1% formic acid in methanol and the following gradient elution (min)/(% B): (0/0), (3/0), (8/93), (12/0), (15/0) was applied at a flow rate

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of 0.8 mL/min, whereof 0.2 mL were split into the ion source. The quantitation was carried out in multiple reaction monitoring (MRM) at *m/z* ratios of 72 and 75 for acrylamide and [2,3,3-*d3*]-acrylamide, respectively. Specific molecular fragments corresponding to *m/z* 55 and 44 for acrylamide and *m/z* 58 and 44 for [2,3,3-*d3*]-acrylamide were also monitored. The daughter ions were obtained through fragmentation with the following conditions: the source temperature was set at 350 °C, nitrogen was used as nebulizer gas at a flow rate of 12 L/h, and the needle and cone voltages were set at 3.0 kV and 100 V, respectively. Under the above-mentioned chromatographic conditions the acrylamide and its corresponding deuterated standard eluted at 7.4 min. For LC/MS/MS analysis acrylamide was quantified using a linear calibration curve built in the range 50−500 ng/mL, the LoD and LoQ were 10 and 20 ng/mL for acrylamide and [2,3,3-*d3*]-acrylamide, respectively and the coefficient of determination r^2 was higher than 0.99.

Statistical analysis

All of the analyses were performed in triplicate both for HRMS and for MS/MS; the results were reported as ng/g of samples for cookies, French fries and ground coffee or as ng/mL for brewed coffee or acrylamide solution. For HRMS, data were recorded using Xcalibur software version 2.1 (Thermo Fisher Scientific). Instead for MS/MS data, Analyst version 1.4.2 (Applied Biosystems, Carlsbad, CA) was used.

Results and discussion

Method development

Although many advances have been performed in acrylamide analysis, improvements in high-throughput quantitative procedures for its determination are still useful. The key point of this paper was to evaluate the potentialities of HRMS in the detection of this small amide in a complex mixture riding out the problems associated to the solid phase extraction and, possibly, to the use of internal standard[13, 14].

The first step of the work dealt with the optimization of chromatographic separation of acrylamide in combination with HRMS detection. It is well known that acrylamide is a very polar molecule with poor retention ($k' < 2.0$) in conventional LC reversed phase sorbents[16, 33]. Four columns with different selectivity were tested evaluating chromatographic performances, including retention time and reproducibility. Among the tested columns, including HILIC phase, pentafluorophenyl phase, and a core shell C18 phase, a polar endcapped stationary phase was selected in order to get a good reproducibility and avoid the interferences due to the solvent, to the matrix and to the unretained

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compounds that eluted along with the solvent front. A typical chromatogram of acrylamide standard is presented in **Figure 1**. Along with polar endcapped column, the use of a core shell C18 phase can ensure a significant reproducibility for several subsequent injections even if the *k'* was always 1. From the chromatographic separation point of view relevant results were obtained with HILIC phase, even if the use of acetonitrile interfered with the ionization of acrylamide.

Although the retention time was quite short (2.85 min), it ensured the separation from the first eluting impurities, the peak shape was maintained, and the deviation of retention time was < 0.1 min among assays of calibration standards, real samples, and recovery samples.

The following step of the experimental work was the HRMS optimization downstream the U-HPLC separation. Specifically, the use of auxiliary gas coupled to a capillary voltage higher than 4.0 kV caused the fragmentation of acrylamide in source. As a consequence it was decided to use an auxiliary gas flow rate of 6 AU in order to avoid undesired fragmentation of the molecules and the formation of the typical fragment (72→55)[26-28, 34]. The lens voltage was optimized setting the mass on 72.0 by manual tuning. This procedure was repeated in two different conditions: direct infusion of the acrylamide standard solution in the ion source and its infusion in the liquid chromatography flow in order to evaluate the interferences of the solvents. The heather temperature and the capillary temperature were optimized to ensure the best solvent nebulization. Under the conditions stated in the method session, Orbitrap MS analyzer showed the best compromise between selectivity and quantitative performance.

Method performance

The method performance was tested against quality parameters such as specificity, carryover, linearity of the calibration, coefficient of correlation (r^2), limit of quantitation, limit of detection, precision, accuracy and recovery. The first point was checking for the absence of any contaminants with an exact mass similar to the one of acrylamide injecting several times pure water. Subsequently a calibration curve was built after determining the LoD and the LoQ. The LoD was calculated according to Armbruster et al [35]. After twenty replicates the limit of blank was 0.5 ppb, the limit of detection was 2.65 ppb and the limit of quantitation was 5 ppb. Two different series of calibration curves were prepared: one spiked with the deuterated internal standard and another one without internal standard. For both series the linearity was achieved in the range 5-500 ng/mL and the r^2 was always higher than 0.99. Each point was injected three time each day and for three consecutive days. After each replicates a blank sample (pure water) was injected in order to verify the absence of carry-over effect. The precision of the two methods was verified in the linearity

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range through the evaluation of the slope of three different calibration curves; the results, reported as %RSD, were lower than 8% for both calibration curves; the accuracy of the method, calculated through the ratio observed amount/specified amount x 100, was always higher than 90%. The recovery test was performed using two different concentration of [2,3,3-d_3]-acrylamide, (100 ng/mL and 253 ng/mL final concentration, n=6 for each matrix) in order to verify the matrix effect and the ion suppression. First the ionization performance of [2,3,3-d_3]-acrylamide was evaluated through the injection of the pure standard dissolved in water and then the recovery was calculated as the ratio between area internal standard and the area of spiked samples. The results ranged from 90% to 99% and they are shown in **Table 2**. In **Figure 2** the recovery of internal standard spiked in French fries is presented. The set-up of the condition for mass accuracy optimization was one of the prominent point of the paper. In a first set of fine tuning trials several lock masses were used to recalibrate the instrument and reduce the mass error[31] and the mass to charge ratio of some contaminants or interference ions was evaluated (**Table 1**), such as methanol ($[M_2+H]^+$,exact mass: 65.05971); methanol/water ($[A_2B_2+H]^+$, exact mass: 101.08084); sodium adduct of methanol/water ($[A_2B_2+Na]^+$, exact mass: 123.06278). Then, 1 µL of [2,3,3-d_3]-acrylamide solution ($[M+H]^+$,exact mass:75.06322) was directly infused in the liquid chromatography flow with a final concentration of 3 µg/mL. The former trials gave unsatisfying results with a mass accuracy around 5 ppm, while the latter trial, which used deuterated acrylamide straight in the calibration solution reduced the mass accuracy to -0.7 ppm. The calibration mixture was spiked with 10 µg of [2,3,3-d_3]-acrylamide in order to set the instrument for the optimal detection of small molecules with an *m/z* lower than 80. The presence of [2,3,3-d_3]-acrylamide in the calibration solution determined the reduction in the discrepancies between the observed mass ($[M+H]^+$,exact mass:72.04434) and the theoretical mass of acrylamide ($[M+H]^+$,exact mass:72.04439).

Measurement of acrylamide content in foods

As it is described above, three of the most commonly consumed acrylamide-containing food, such as French fries, coffee and cookies, were selected to test the performance of the developed method. The determination of acrylamide in these food matrices showed different requirements in terms of extraction and clean-up before chromatographic separation[14]. Up to now acrylamide was detected through solid phase extraction along with liquid chromatography and tandem mass spectrometry detection[28, 34, 36-38]; by LC/MS after derivatization with 2-mercaptobenzoic acid[15] ,by direct gas chromatography coupled to mass spectrometry[39] and by pyrolysis GC/MS[40]. The extraction procedure used in this paper was exactly the same for cookies, French fries and ground coffee while for brewed coffee only a dilution in water was performed. The recovery test and the LoD were

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evaluated in the different matrix, with satisfactory results as presented in **Table 2,** where the LoD and LoQ in the tested foods and in standard solutions were almost similar**.** In **Table 3** acrylamide concentrations obtained on the various foods with and without the use of internal standard were reported and they were compared not only with those present in the literature, but also with the results obtained by LC/MS/MS on the same samples. For French fries the value ranged from 270 ng/g ± 12.1 ng/g (254 ± 4.6 ng/g for internal standard analysis) to 424 ± 10.3 ng/g (490 ± 10.0 ng/g for internal standard analysis) according to the processing time (five and seven minutes respectively)[41]. These values were similar to the ones obtained through LC/MS/MS as reported in **Table 3**[27, 42, 43]. For cookies samples two different typologies of cookies prepared *de novo* according to two recipes, already used in a previous paper from our group, were used for acrylamide determination[32]. The value varied from 276 ± 3.5 ng/g (cookies with 0.65% sodium chloride) to 317 ± 9.2 ng/g (cookies without 0.65% sodium chloride). The use of the internal standard resulted in similar values: 262 ± 4.0 ng/g and 340 ± 5.6 ng/g for cookies with sodium chloride and without, respectively. These data clearly showed the substantial agreement between the U-HPLC/HRMS method and the SPE LC/MS/MS method **(Table 3)**.

The method performances were tested also in brewed coffee sample and in ground coffee. In this case the acrylamide content was 12.1 ± 0.1 ng/mL and 140 ± 3.6 ng/g for brewed coffee and ground coffee, respectively and the previous results were fully confirmed[44-47], even if with the use of [2,3,3-d_3]-acrylamide small differences were present: 15 ± 0.2 ng/mL and 159 ± 8.1 ng/g for brewed coffee and ground coffee, respectively. The key points for brewed coffee were the LoD and the recovery that were 3 ppb and 90%, respectively. Acrylamide detection in coffee is characterized by several drawbacks due to the matrix effect and to the roasting of coffee beans[44]. For this reason for LC/MS/MS the use of internal standard is a key point that can be easily overcome thanks to the HRMS. This was presented in **Table 3** where the analysis performed through HRMS or MS/MS showed similar results.

The results obtained with the developed methods were in any case well in line with those present in literature[48, 49]. From the analytical point of view the use of [2,3,3-d_3]-acrylamide increased the robustness of the method, in particular the variability due to the ion suppression or to the matrix effect were eliminated for each analysis. Interestingly without the use of labeled internal standard the results were still satisfying and the differences between the two methods were less than 14% in solid foods, thus suggesting that in the cases not requiring a high precision the use of HRMS method could theoretically allow also to avoid the use of external standard.

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A huge variety of papers has been published on quantitation procedure for acrylamide in foods. The method here developed using U-HPLC Orbitrap HRMS proved to be very effective as it allowed to ride out the matrix effect and the time consumption due to solid phase extraction. Moreover the use of a modified calibration solution allowed the optimal set of the instrument and it improved enormously the mass accuracy for acrylamide detection. Also the use of internal standard could be theoretically avoided with a negligible loss in method accuracy. From the analytical standpoint the performances were satisfactory: the relative standard deviation was less than 7%, the recovery in the three different matrix was always higher than 90% and the limit of detection was comparable to the ones of tandem mass spectrometry.

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Monoisotopic ion mass	Ion Type	Formula for M	Compound ID	Origin
65.05971	$[M_2+H]^+$	CH_3OH	Methanol	Solvent
101.08084	$[A_2B_2+H]^+$	$[MeOH]_n[H_2O]_m$	Methanol/water	ESI solvents
123.06278	$[A_2B_2+Na]^+$	$[MeOH]_n[H_2O]_m$	Methanol/water	ESI solvents
391.28429	$[M+H^+]$	$C_{24}H_{38}O_4$	diisooctyl phtalate	Plasticizer
75.06322	$[M+H^+]$	$C_3H_2D_3NO$	[2,3,3-d_3]-acrylamide	External infusion

Table 1: Contaminant or interference ions used as locking mass to recalibrate the instruments during the run[31]

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	RT (min)	LOD (ppb)	LOQ (ppb)	Recovery (%)	% RSD	%RSD*	Linearity
French fries	2.85	2,65	5	97	8	9	
Brewed coffee	2.83	3	5	90	7	9	
Ground Coffee	2.85	3	5	90	7	5	
Cookies	2.85	2,65	5	92	6	5	
Standard	2.83	2.65	5	/	< 7	<5	>0.99

Table 2 LC/HRMS performances for acrylamide detection in three different foods. %RSD* indicates the set of samples performed with labeled internal standard

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Food	No labeled standard	Labeled standard	LC/MS/MS	Literature data	Ref.
Cookies (different formulations)	276 ± 3.5 ng/g 317 ± 9.2 ng/g	262 ± 4.0 ng/g 340 ± 5.6 ng/g	286 ± 15.3 ng/g 325 ± 9.3 ng/g	278-313 ng/g	*32*
				451-510	*15*
French fries (different frying time)	270 ± 12.1 ng/g 424. ± 10.3 ng/g	254 ± 4.6 ng/g 490 ± 10.0 ng/g	280 ± 18.8 ng/g 409 ± 11.1 ng/g	253-2688	*42*
				150-1200	*43*
				50-1800	*27*
Brewed coffee	12 ± 0.1 ng/mL	15 ± 0.2 ng/mL	13 ± 0.2 ng/mL	6-16 ng/mL	*46*
				5-12	*44*
				14-21	*45, 49*
Ground coffee	140 ± 3.6 ng/g	159 ± 8.1 ng/g	145 ± 3.2ng/g	45 – 374 ng/g	*46*
				267; RSD %: 0.5	*47*

Table 3 Acrylamide concentration in the tested foods, through LC/HRMS (with and without labeled internal standard); through LC/MS/MS and literature data.

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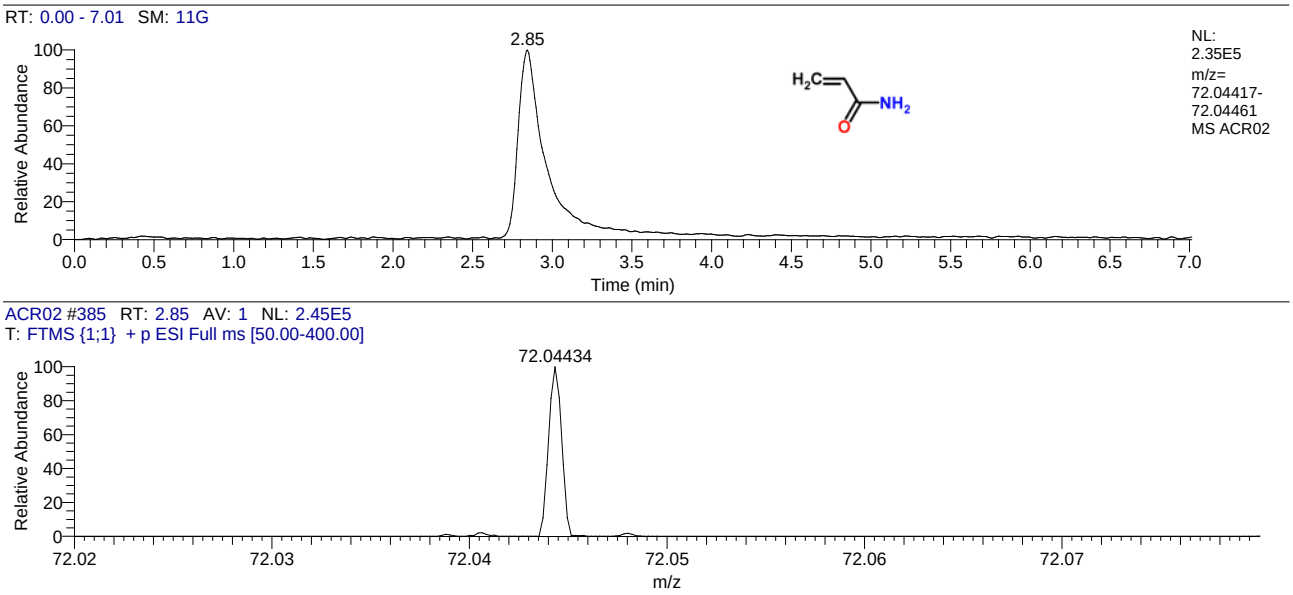

Figure 1 Typical extracted ions chromatogram and mass spectrum of acrylamide standard dissolved in water. Δ ppm = -0.7. Acrylamide theoretical mass: 72.04439**.**

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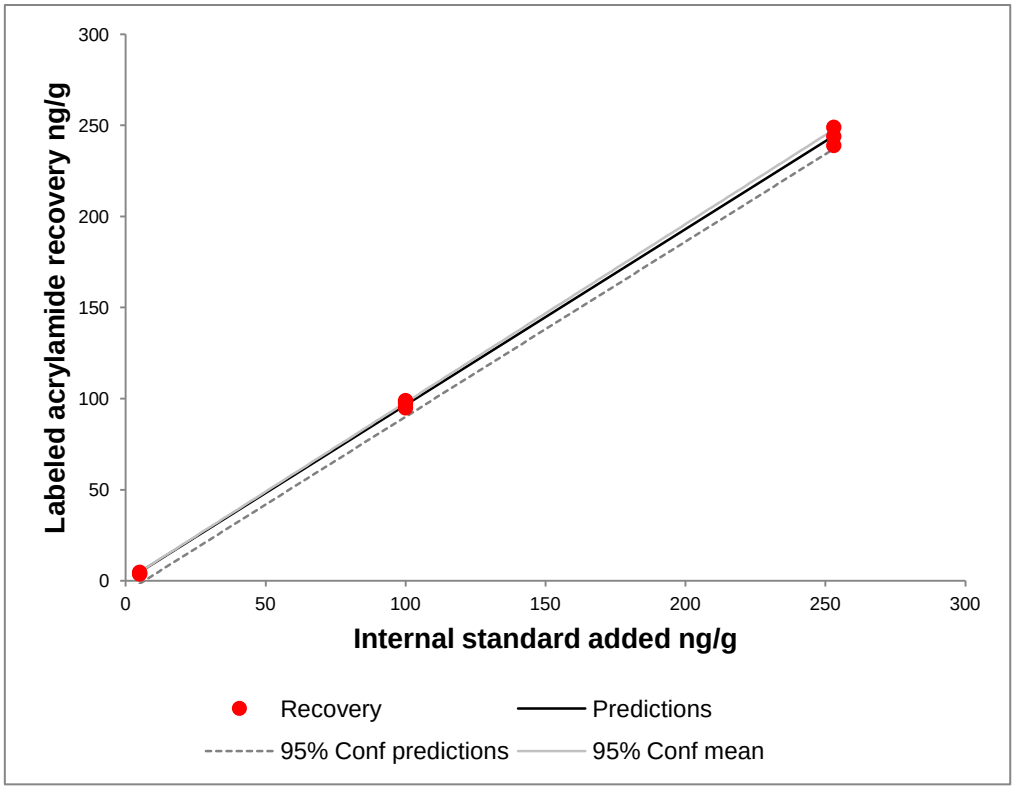

Figure 2 Recovery of internal standard spiked in French fries. The dotted lines represent the 95% confidence intervals calculated on the predictions and on the mean (n=3)

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