New tools in modulating Maillard reaction from model systems to food

Antonio Dario Troise
Thesis committee

Promotor
Prof. Dr V. Fogliano
Professor of the Food Quality and Design
Wageningen University

Co-promotors
Dr C.C. Berton-Carabin
Assistant professor, Food Process Engineering Group
Wageningen University

Prof. Dr P. Vitaglione
Associate professor, Human Nutrition
University of Naples, Italy

Other members
Prof. Dr P. Schieberle, Technical University of Munich, Germany
Prof. Dr B. De Meulenaer, Gent University, Belgium
Prof. Dr H. Schols, Wageningen University
Dr P. Dekker, DSM Food Specialties, Delft

This research was conducted under the auspices of the Graduate School of Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences.
New tools in modulating Maillard reaction from model systems to food

Antonio Dario Troise

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 27 October 2015
at 1.30 a.m. in the Aula.
Antonio Dario Troise

New tools in modulating Maillard reaction from model systems to food,
127 pages.


With references, with summary in English

A Daniele, grazie.
**Table of contents**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>3</td>
</tr>
<tr>
<td>General Introduction</td>
<td>3</td>
</tr>
<tr>
<td>The Chemistry of Maillard reaction in a nutshell</td>
<td>4</td>
</tr>
<tr>
<td>Maillard reaction products and their implication in food quality</td>
<td>5</td>
</tr>
<tr>
<td>The Maillard reaction in foods: desired and undesired effects</td>
<td>6</td>
</tr>
<tr>
<td>The control of the Maillard reaction as a tool for the promotion of food quality</td>
<td>11</td>
</tr>
<tr>
<td>Aim of the thesis</td>
<td>13</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>17</td>
</tr>
<tr>
<td>Reactants encapsulation and Maillard reaction</td>
<td>17</td>
</tr>
<tr>
<td>Introduction</td>
<td>19</td>
</tr>
<tr>
<td>MR and mitigation strategies</td>
<td>20</td>
</tr>
<tr>
<td>Encapsulation: current use in foods</td>
<td>22</td>
</tr>
<tr>
<td>Modulating Maillard reaction through encapsulation</td>
<td>25</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>26</td>
</tr>
<tr>
<td>Iron and metal ions</td>
<td>27</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>28</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (PUFA)</td>
<td>29</td>
</tr>
<tr>
<td>Conclusion and future perspectives</td>
<td>30</td>
</tr>
<tr>
<td>Faox enzymes inhibit development of the Maillard Reaction in storage both in the of protein glucose model system and low lactose UHT milk</td>
<td>33</td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>36</td>
</tr>
<tr>
<td>Expression and purification of Faox I and II</td>
<td>36</td>
</tr>
<tr>
<td>Enzymatic Activity</td>
<td>37</td>
</tr>
<tr>
<td>Circular Dichroism</td>
<td>37</td>
</tr>
<tr>
<td>Multiple-Angle Light-Scattering (MALS) analysis</td>
<td>37</td>
</tr>
<tr>
<td>Experimental systems</td>
<td>38</td>
</tr>
<tr>
<td>Evaluation of Maillard Reaction development in the model system</td>
<td>38</td>
</tr>
<tr>
<td>Quantification of b-HMF</td>
<td>38</td>
</tr>
<tr>
<td>Quantification of Ne-(Carboxymethyl)-L-lysine (CML)</td>
<td>39</td>
</tr>
<tr>
<td>Evaluation of protein glycation on the β-LG-glucose model system</td>
<td>40</td>
</tr>
</tbody>
</table>
Reduction and carboxymethylation procedure ................................................................. 40
Protein hydrolysis ............................................................................................................. 40
MALDI-TOF analysis .......................................................................................................... 40
Statistical analysis ............................................................................................................... 41
Results and discussion ....................................................................................................... 41
Biochemical characterization of Faox I and Faox II .............................................................. 41
Faox enzymes reduce the formation of CML in low lactose milk (LLM) and protein glucose model system .............................................................................................................................. 42
Faox enzymes reduce the formation of bound hydroxymethylfurfural (b-HMF) in low lactose commercial milk and protein glucose model system .......................................................................................................................... 44
The peptides of b-LG represent a marker to monitor the Faox activity ................................ 46
Conclusions ............................................................................................................................. 49
Chapter 4 .................................................................................................................................. 51
Effect of olive mill wastewaters phenol compounds on reactive carbonyl species and Maillard Reaction end-products in ultra-high temperature treated milk .................................................................................................................. 51
Introduction ............................................................................................................................... 53
Material and methods .......................................................................................................... 55
Chemicals ................................................................................................................................. 55
Formulation of OMW .............................................................................................................. 55
OMW characterization .............................................................................................................. 56
Lab scale UHT Milk treatment ................................................................................................. 56
CML, total lysine and furosine analysis .................................................................................. 57
Free Amadori products ........................................................................................................... 58
Quantification of reactive carbonyl species (RCSs) ............................................................. 58
Quantification of Maillard related off-flavor volatiles ............................................................ 59
Statistical analysis .................................................................................................................. 59
Results and discussion .......................................................................................................... 60
OMW characterization and thermal treatment ........................................................................ 60
Off-flavor in UHT milk ............................................................................................................ 61
Trapping activity towards RCSs ............................................................................................. 62
Amadori compounds reduction ............................................................................................. 65
Control of bound MRPs formation .......................................................................................... 66
Conclusions ............................................................................................................................. 68
Chapter 5 .................................................................................................................................. 69
Amadori products formation in emulsified systems .............................................................. 69
Introduction ........................................................................................................................................... 71
Material and methods .............................................................................................................................. 73
  Chemicals and reagents ......................................................................................................................... 73
  Model systems preparation .................................................................................................................. 73
  Thermal treatment ............................................................................................................................... 74
Emulsions characterization ...................................................................................................................... 75
Microscope analysis ............................................................................................................................... 76
Amadori compounds and amino acids analysis .................................................................................. 76
D-Glucose analysis ............................................................................................................................... 77
Statistical analysis ................................................................................................................................. 78
Results and discussion ........................................................................................................................... 78
  Process setup ....................................................................................................................................... 78
  Physicochemical characterization of emulsions .................................................................................. 78
  Amadori compounds formation by HRMS analysis ........................................................................... 80
Conclusions ............................................................................................................................................ 84
Chapter 6 ............................................................................................................................................... 87
General discussion ................................................................................................................................. 87
  Introduction ......................................................................................................................................... 88
  Snapshot of thesis main achievements .............................................................................................. 92
Impact of the results on academy and industry ..................................................................................... 98
Future recommendation and forthcoming strategies: a) tuning reactants location ......................... 100
Future recommendation and forthcoming strategies: b) the chemometric approach ..................... 103
Promoting the quality of processed foods through the Maillard reaction ....................................... 104
Summary .............................................................................................................................................. 107
References ........................................................................................................................................... 109
Overview of completed training activities .......................................................................................... 124
List of publications ................................................................................................................................ 125
Acknowledgements ............................................................................................................................... 127
Abstract

The Maillard reaction (MR) supervises the final quality of foods and occupies a prominent place in food science. The first stable compounds, the Amadori rearrangement products (APs) and Heyns rearrangement products (HPs), represent the key molecules from which a myriad of reactions takes place and each of them contributes to the formation of Maillard reaction end-products (MRPs) or advanced glycation end products (AGEs).

Several papers have dealt with the control of the MR in foods ranging from the thermal loading reduction, to the use of alternative process technologies, reactants impact or enzymes, as well as to the monitoring of the end-products formation by multiresponse modeling. The strategies used up to now aim at common goals: the reduction of potentially toxic compounds and the promotion of desired molecules formation as well as flavor, aroma, color and texture attributes. In other words the ultimate target is the promotion of food quality by tuning the MR.

This thesis introduces four alternative strategies that are able to control the final extent of the MR in foods.

- The possibility to segregate reactants by encapsulating some minor components and thus delaying the MR was highlighted in Chapter 2. The encapsulation of sodium chloride, ascorbic acid, PUFA and iron inside hydrophobic capsules was used as a possible example: the core material release over the time delayed the reaction rates.
- The results obtained through the treatment with the enzyme fructosamine oxidase (Faox) I and II which is able to deglycate free Amadori products and capitalize the local unfolding of lysine peptide bound residues were reported in Chapter 3. Data showed that Faox can reduce the formation of Ne-(Carboxymethyl)-l-lysine and bound hydroxymethylfurfural in model system and in low lactose milk.
- The effects obtained with the addition of spray-dried olive oil mill wastewaters in milk was illustrated in Chapter 4. This ingredient acts as a source of phenylethanoids, which can trap α-hydroxycarboxyls and α-dicarboxyls and can form adducts with amino groups after the oxidation of phenolic rings into quinone. The use of this functional ingredient before milk thermal treatment resulted in a reduction of off-flavor, reactive carbonyls species and bound MRPs.
• The possibilities offered by the location of MR reactants in microemulsion was investigated in Chapter 5. The oil/water partition coefficient of amino acids played a key role in the formation of Amadori compounds. The anchoring effect of tricaprylin and Tween 20 toward aliphatic amino acids in microemulsion systems was evaluated and compared to a control aqueous solution of amino acids and glucose. Results confirmed the hypothesis: the higher the partition coefficient the lower the formation of aliphatic amino acids Amadori compounds.

All of the four proposed strategies involved location and interaction of reagents, reactants, intermediates and final products. As a result each strategy depicted a specific route for the control of the final extent of the MR. Many steps are still necessary to scale up these methodologies into the food production chain, however new ways for obtaining foods of superior quality have been paved.
Chapter 1

General Introduction
General Introduction

In this chapter the background for the evaluation of four novel strategies for the control of the Maillard reaction (MR) in foods is presented. It offers the guidelines to understand the implication of these strategies on the fate of precursors. Moreover, it illuminates the relationship between MR, foods, previous mitigation strategies and their effects on Maillard reaction end products (MRPs). The questions behind the present research, i.e. the use of encapsulation, deglycating enzymes, spray dried olive oil mill wastewaters as well as microemulsions in the tuning of the MR are highlighted with special emphasis on chemical aspects and final food quality.

The Chemistry of Maillard reaction in a nutshell

The Maillard reaction (MR) is defined as “an array of non-enzymatic, consecutive, parallel chemical reactions” that supervises the final quality of foods. Despite its complexity, the MR requires the presence of reducing carbonyls, primarily carbohydrates, and amino compounds of biological origin. Since Louis-Camille Maillard first observed in 1912 that a mixture of amino acids and sugars resulted in a brown solution upon heating, overwhelming evidences establishing the condensation reaction between reducing sugars and amino groups of free amino acids or proteins has been the main source of D-fructosamine derivatives in foods and in vivo. The initial condensation of free D-glucose and amino acids leads to the formation of a labile N-substituted D-glucosylamine which may undergo the Amadori rearrangement to form the respective N-substituted D-fructosamine, an 1-amino-1-deoxy-2-ketose (AP). The specular mechanism takes place in presence of D-fructose: in this case the formation of an unstable N-substituted D-fructosylamine occurs, subsequently, upon the Heyns rearrangement to the N-substituted D-glucosylamine, an 1-amino-2-deoxy-1-aldhose (HP) is formed. The initial stage of the MR, also known as activation stage, can be summarized by the Amadori and Heyns rearrangements. The relatively stable APs and HPs can react essentially following two paths: 1,2-enolisation via 3-deoxy-1,2-dicarboxyls, 2,3-enolisation via 1-deoxy-2,3-dicarboxyls, the choice being mainly affected by pH, a low pH favoring 1,2-enolisation and vice versa.

According to the compounds formed, the Maillard network can be outlined as such:

A) the blockage of free and bound amino acids with the consequent reduction in the nutritional values of foods. The consequence is the formation of compounds with potential mutagenic
properties and compounds that can cause cross-linkage of proteins. Reactions of this type apparently also play a role in vivo;

B) the fragmentation, conversion and isomerization of the APs and HPs that along with cyclization, Strecker’s aldehyde formation and degradation, retro-aldol condensation via Namiki pathway, pyrolysis, oxidative cleavage and polymerization are linked to the formation of volatile compounds, reductones and dicarbonyls. These substances include also flavoring matter, especially bitter molecules;

C) the formation of brown pigments, known as melanoidins, which contain variable amounts of nitrogen, molecular weights and their solubility in water depends on the structural rearrangement. The structure of these compounds has not been resolved yet.4

These pathways can be combined in concordance with the pH, temperature, pressure and water activity, while precursors and intermediates can react with each other in presence of amino acids following one or more of the mechanisms to form different classes of molecules.5

**Maillard reaction products and their implication in food quality**

Despite a clear distinction among the several routes of the MR is not so easy, it is widely accepted that several interactions can lead to the formation of undesired and desired molecules. The Maillard derived volatile compounds, the MRPs arising from the amino acids blockage, and the brown/yellow pigments of melanoidins represent the most studied classes.

A tentative classification of MR volatiles has been proposed by Nursten:5

- “Simple” sugar dehydration/fragmentation products: furans, pyrones, cyclopentenes, carbonyl compounds, acids;
- “Simple” amino acid degradation products: aldehydes, sulphur compounds (e.g. hydrogen sulphide, methanethiol), nitrogen compounds (e.g. ammonia, amines);
- Volatiles produced by further interactions: pyrroles, pyridines, pyrazines, imidazoles, oxazoles, thiazoles, thiophenes, dithiolanes, thithianes, dithianes, thithianes, furanthiolos.

Overlooking the technological functionality of glycated amino acids and proteins, i.e. the improved solubility and color, the presence of blocked amino acids implies not only a loss in the nutritional value, but also the presence of potentially toxic outcomes. The reaction between reducing sugars or carbonyls and the nucleophilic side chains of amino acids, in particular the ε-amino group of
lysine of the guanidino side chain of arginine promotes the formation of pyrraline, pentosidine, glucosepan, hydroimidazolones, Ne-(Carboxymethyl)-L-lysine (CML), Ne-(Carboxyethyl)-L-lysine (CEL) and lysino-alanine. Since lysine is an essential amino acid, its ε-amino group has special significance: the reaction with glucose gives N-ε-deoxyfructosyllysine that after acidic hydrolysis is converted into furosine (20%) and pyridosine (10%) and lysine (50%). Moreover, CML and CEL can form either from the Schiff base (via Namiki pathway) or by Baeyer–Villiger oxidation of Amadori products. In particular CML and CEL along with furosine are the most extensively used markers of thermal treatment in milk based products. On the contrary, the study of free marker of advanced stages of the MR involve the monitoring of HMF and acrylamide. The main precursor of acrylamide is asparagine that can be thermally decomposed by deamination and decarboxylation in presence of reducing sugars via Strecker degradation where the last intermediates is represented by acrolein or 3-aminopropionamide. HMF is a furanic compound that can be formed both from MR both from caramelization and autoxidation of sugars, via 3-deoxyosone formation.

![Figure 1.1: Desired molecules (green) and undesired molecules (red).](image)

**The Maillard reaction in foods: desired and undesired effects**

Food quality is the direct consequence of two aspects: the ability to satisfy the sensory expectations of consumers and the absence of compounds with potential negative effects on health. The concentration of MRPs and their effects are the result of the interaction and location
of precursors, according to time, temperature, pH and water activity. The distinction between desired molecules and undesired molecules is specific to each food and the relationship between processes, formed substances and physicochemical modifications is the central aspect of the final quality of foods.

Focusing on the processes, roasting, baking or frying all lead to favorable effects of the Maillard reaction such as color and flavor formation while during drying, pasteurization and sterilization the occurrence of the Maillard reaction is unwanted with the simultaneous formation of undesired molecules and the loss of nutrients. Generally, the relationship between process and formed molecules is complex and it needs to be evaluated time by time in relationship to the matrix: the typical example is the frying process in potato. On the one hand the production of volatiles is particularly desired along with the color development on the surface, but on the other hand the presence of asparagine and reducing sugars at temperatures above 100 °C promotes the formation of acrylamide via Strecker degradation of fructose-asparagine and 3-aminopropionamide formation.

The effects of MR on different food classes is reported in Table 1.1 along with some of the mitigation strategies proposed.
Table 1.1: MR in foods, classes of compounds formed and mitigation strategies proposed

<table>
<thead>
<tr>
<th>Foods</th>
<th>Undesired molecules</th>
<th>Mitigation strategies</th>
<th>Desired molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy products</td>
<td>2-Acetyl-2-thiazoline, methional, o-aminoacetophenone, Dicarbonyls, CML, CEL, GOLD, DOLD, MOLD, argpyrimidine, glucosepan, APs, CMA, pyrraline, pentosidine, Hydroxycarbonyls</td>
<td>Thermal loading reduction Antioxidants/Polyphenols Thermal loading reduction, deglycating enzymes, encapsulation of reactants</td>
<td>Volatile compounds</td>
</tr>
<tr>
<td>Bakery products</td>
<td>HMF, Acrylamide, Furan, Mutagenic compounds, Heterocyclic amines</td>
<td>Polyphenols Thermal loading, ohmic heating, vacuum, encapsulation Antioxidants, bivalent ions addition, free amino acids addition</td>
<td>Volatile compounds, Brown/yellow pigments</td>
</tr>
<tr>
<td>Meat</td>
<td>Mutagenic compounds, Heterocyclic amines</td>
<td>Thermal loading, alternative technologies, salts and bivalent ions effects</td>
<td>Mercaptoaetaldehyde, acetaldehyde, hydrogen sulphide, pyridines, pyrazines, Reductones, Color, Yellow pigments</td>
</tr>
<tr>
<td>Potato</td>
<td>Acrylamide</td>
<td>Asparaginase, low asparagine, thermal loading, ions addition,</td>
<td>Brown pigments, aroma, melanoids</td>
</tr>
<tr>
<td>Coffee</td>
<td>Acrylamide</td>
<td>Alternative technologies</td>
<td>Diketopiperazines, bitter compounds, pyrazines</td>
</tr>
<tr>
<td>Cocoa and tea</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dairy products are subject to the MR where lactose and lysine residues, mainly caseins are the principal reactants. The consequences of the thermal treatment on milk, milk based products and infant formulas can be summarized as follows: blockage of lysine residues with the reduction of their availability; formation of volatiles (low molecular weight fission products) as a consequence of the degradation of the APs; formation of antioxidative and antibacterial compounds; synthesis of mutagenic, antimutagenic compounds, polymerization of proteins and brown/yellow color development (according to the water activity and to the thermal loading). In dairy products the most used markers of the thermal treatment are furosine, formed during acid hydrolysis of the protein-bound Amadori product of lysine, and other lysine derivatives such as CML, CEL, pentosidine, pyridosine and lysinoalanine. Also dicarbonyls (mainly glyoxal, methylglyoxal and 3-deoxyglucosone) and off-flavor (sulphur derived volatile compounds and diacetyl) are used as
markers of the thermal treatment even if their highly reactivity represents a challenging outcomes.

In bakery products the external aspect, color surface and flavor are the most important quality parameters for their acceptance. The main changes supervised by the MR refer to structural modifications where the polymerization of proteins and sugars leads to the formation of brown nitrogenous polymers and copolymers typical of the crumbs. The formation of volatiles is mediated by the degradation of the APs, HPs and to the interaction of precursors and intermediates in the advanced stage of the reaction. In bakery products, where the MR proceeds hand in hand with sugar caramelization, the widely used markers of the thermal treatments are HMF, furan, dicarbonyls and acrylamide. Acrylamide and HMF are considered as probably or potentially carcinogenic to humans or might be metabolized by humans to potentially carcinogenic compounds. Both are mainly formed through MR, even if HMF can derive also from the direct pyrolysis of sucrose, and they can be regarded as the most important heat-induced contaminants occurring in bread and bakery products.\(^7\)

In meat products the MR depends on the thermal loading; it is typical of roasting where the formation of bitter substances (reductones and dehydroreductones) and volatiles are among the most required aspects by consumers. Specifically the aroma pattern is characterized by several molecules whose concentration depends on the type of precursors and conditions: they are primarily caused by the Strecker aldehydes methyl propanal, 2-and 3-methyl butanal as well as the typical roast aroma substances 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline and the two alkyl pyrazines. The thiazoline and the pyrroline are also formed in lower concentrations during the boiling of meat, as well. 2-Acetyl-2-thiazoline and alkylpyrazines are the most important roast aroma substances in fried meat, with the former lasting for a limited amount of time, the latter lasting longer. Beside the formation of volatiles, pyrolyzates of amino acids and proteins are responsible for mutagenic effects in microbial tests, where the presence of heterocyclic amines, such as pyridoinoindoles, pyridoimidazoles, tetra-azafluoroanthenes, imidazoquinolines and imidazoquinoxalines is one of the most stressed health concerns. All these compounds are formed via the MR in presence of creatinine and reducing carbonyls.

In coffee, the roasting is the crucial process where temperatures from 100 °C to around 200 °C promotes significant modifications in the green beans: increase in volume, decrease in the specific gravity, evaporation of water with a final moisture content of 1.5-3-5 %, loss of weight and the MR
related brown color development, melanoidins and aroma formation. The volatiles formed during the roasting of coffee are as complex as the MR network: more than 850 substances have been identified until now. Melanoidins are present in the soluble fraction of roasted coffee. Their molecular weight ranges from 4 up to 10 kDa and can derive both from MR and carbohydrate caramelization. The hydroxycinnamic derivatives, such as chlorogenic acid and caffeic acid are also involved in such browning reactions since caffeic acid has been identified in alkali hydrolysates of melanoidins. Along with the volatiles identification, in coffee products the most important marker used is acrylamide: the presence of free amino acids and specifically of asparagine, reducing sugars and the high temperatures lead to the formation of this compounds.

Similar to coffee, cocoa and tea can be influenced by the MR. The fermented tea leaves are dried with hot air at ca. 90 °C to a water content of 3 to 4%. In this process the leaf material is heated to 80 °C, which is sufficient to inactivate the polyphenol oxidases. This process governs the formation of volatiles, along with the degradation of APs and HPs, the oxidation of the numerous phenolic compounds can limit the effect of dicarbonyls. The chemical mechanisms involve 1,3-disubstituted rings where the epicatechin – dicarbonyls adducts are presumably generated by hydroxyalkylation and aromatic substitution reactions. These mechanisms shall be discussed later on in Chapter 4. In fermented cocoa beans the roasting process reduced the moisture content up to 3% where undesired components such as acetic acid, oxidized phenolic compounds and esters are partially removed. This process crucially contributed to the development of the typical aroma pattern and the most important concentrated odorants are: acetic acid, 3-methylbutanal, 2-methylbutanal, phenylacetaldehyde, 3-Methylbutyric acid, 2-phenylacetic acid and 2-methylbutyric acid, while for the bitter taste, apart from theobromine, diketopiperazines are the most important compounds.14

The MR occurs in other vegetable products and its effects are particularly important in brewing. Even if the contribution in the odorants is also mediated by the presence of hops, dark caramelized malt is obtained by roasting up to 190°C – 220°C according to the desired color and other properties such as foaming and full bodied-properties.

Each food represents a complex matrix and the control of desired and undesired molecules formation is a challenging outcome. The specific design, the optimization and the control of the above-mentioned processes for the production of food with the desired quality and stability can represent the optimal answer to consumer needs.
The control of the Maillard reaction as a tool for the promotion of food quality

The control of the MR is still one of the hottest topic of Food Chemistry and its implication in the promotion of food quality is one of the most stressed aspects in the Academic research and in the food industry. Inhibition of the MR in foods is important for three reasons: in cases where color formation is undesirable, its results are unsightly; in cases where browning flavors are undesirable; in cases where the concentration of precursors can lead to the formation of harmful compounds.

The control of MR is often synonym of “control of color formation” and several strategies have been optimized in order to guarantee this attribute without influencing the formation of volatiles and other potentially toxic molecules. A huge variety of examples is possible: they include the use of vacuum baking, the addition of bivalent cations able to subtract water and increase browning rate, use of several compounds to control the pH. According to Nursten, there are six ways to control or inhibit the extent of nonenzymic browning:5

a) Refrigeration, since MR has a high-temperature coefficient;
b) Use of sulphur dioxide (at concentration up to 1.2%, depending on the food matrix and to legal limits) that increases the lag before the browning starts, the chemical explanation relies on the reversible combination with carbonyls;
c) Low pH, even with the addition of ascorbic acid. Increasing too much the concentration of ascorbic acid browning will be worse than without it;
d) Dehydration is helpful in preventing browning, but the temperature plays a key role, partial dehydration may make browning worse rather than better, especially in presence of high concentration of reducing carboxyls, also the volatiles can be strongly influenced;
e) Removal or reduction of precursors, the typical examples are fermentation, the removal of lactose before drying milk skimmed and the use of oxidase;
f) Removal of amino compounds, i.e. coagulation and filtration of protein. The reduction in the nutritive value is the main drawback for the application of this method

Along with the inhibition of nonenzymic browning, other strategies attempt to modulate volatiles formation and reduction of toxic molecules. The control of the focused on the tune of the time and temperature of the process: the formation of volatiles or the improvements of texture can be optimized taking into account the specificity of the matrices and the thermal loading. In the last decades, beside the growing interests in the potential toxicity of some MRPs, such as acrylamide,
HMF, reactive carbonyls and along with a wide request of healthier foods, several strategies have been introduced. Most of them referred to acrylamide reduction in fried potatoes, bakery products and coffee while some other strategies were imported from the control of the MR in vivo, such as the use of enzymes and trapping agents.\textsuperscript{15} Several strategies have been reviewed up to now: they range from the tune of the time and temperature couple, to the use of alternative technologies (ohmic heating, vacuum, radiofrequencies). The reduction of the thermal loading is possible with a deep study of the mechanistic model of acrylamide formation: the multiresponse modeling is the key to understand the effects of temperature and moisture content, as well as the contribution of asparagine, glucose and fructose.\textsuperscript{16} Specific agronomic interventions have been optimized in order to reduce the concentration of asparagine, whilst one of the most promising tools is the use of asparaginase as a mean to promote asparagine oxidation into aspartic acid. Other mitigation strategies include changing in recipes and formulations addition of proteins, glycine, cysteine and other amino acids more reactive than asparagine, organic acids and acidulants, calcium ions, cyclodextrin, natural antioxidants or antioxidant extracts able to interfere with the formation of intermediates; replacement of reducing sugars with sucrose and of ammonium bicarbonate with sodium bicarbonate and pre-thermal microwave cooking.\textsuperscript{17,18}

The control of flavor development in milk can be regulated by the use of phenolic compounds able to trap dicarbonyls. These mechanisms have been extensively studied by Peterson and Ho’s group with particular emphasis on the chemical insights beside the formation of the adduct epicatechin – methylglyoxal.\textsuperscript{19,20} Other strategies with glyoxalases, ketosamine kinases (fructosamine 3-kinase (FN3K) and fructosamine 3-kinase related protein (FN3K-RP)),\textsuperscript{21} followed an approach similar to the one proposed for Faox I and II.

In chapter 2, an overview of the strategies proposed up to now is reviewed. These techniques are generally characterized by: individuation of the drawback linked to the MR (i.e. formation of off-flavor or toxic molecules), individuation of the potential markers (i.e. flavors, acrylamide or CML), optimization of the strategy (i.e. reducing thermal loading), monitoring of the markers, implementation at laboratory scale then at industry level.

In this thesis strategies able to overall offers tuning the MR rather than pointing at the reduction of a single component or a class of compounds have been investigated. This schema is reported in Figure 2 where along with the introduction of four novel strategies the control of the MR is tackled from a general point of view by monitoring several molecules at different stages. Despite
the fact that the four strategies here reported are clearly different and in some aspects cannot be compared to one another, they are characterized by a common feature: the overall control of the MR is optimized at different stages, i.e. in the reduction of intermediates during the early stage of the reaction or end products at advanced stage.

**Figure 1.2:** Two different approaches in the control of the MR. Left part: previous reported approaches. Right part: overall approach with the four novel strategies and the tentative control of each step of the MR.

**Aim of the thesis**

This thesis moves from the study of the chemical mechanisms beside the formation of MRPs and tries to elucidate the pros and cons of four novel strategies. Specific emphasis is put on the control of the early stage of the MR, with the formation of the APs and its relationship with the formation of off-flavor, reactive carbonyls species and MRPs such as bound HMF and CML.
The following research questions depict the background and the starting point beside the results obtained:

- Is the control of the Maillard reaction related only to the tuning of the thermal loading?
- Which is the role of encapsulation strategies? Only protective/carrier purposes?
- Is low lactose milk the ideal substrate for the control of the MR by enzymatic approach?
- Are the carbonyls trapping and iminoquinone and iminophenol formation also possible for secoiridoids derivatives?
- What is the relationship between the control of the MR and the reactants location?

The following chapters tackle with the above mentioned questions by evaluating in turn the physicochemical relationship between the four approaches and MR. The two key aspects that govern the strategies proposed involve interaction (i.e. between precursors and reactants, enzyme and intermediates) and location (i.e. for the Amadori compounds formation in microemulsion system).

In Chapter 2 the encapsulation will be proposed as a new strategy able to control the formation of MRP. The effect reactants involved in the MR, such as sodium chloride, PUFA, ascorbic acid and iron will be evaluated in their relationship toward HMF, acrylamide, CML and CEL formation. The encapsulation will be not only related to the carrier and protection of the core material, but also to control of a chemical reaction.

In Chapter 3, the use of de-glycating enzymes, such as Faox I and II will be considered as a potential tool for the reduction of the glycation in model systems and low lactose milk with particular attention to the CML, b-HMF formation and to overall peptides glycation.

In Chapter 4, the effect of secoiridoids derivatives, such as tyrosol, 3-hydroxytyrosol and verbascoside will be evaluated in the reduction of reactive carbonyls species, such as α-hydroxycarboxydl and α-dicarboxyls, off-flavor and MRP in UHT milk. A preliminary hypothesis on the reaction mechanisms will be also introduced focusing on oxidation of the phenolic rings and on the dicarbonyls trapping.

In Chapter 5 the effect of microemulsion systems on Amadori compounds formation and the relationship between amino acids partition coefficient and reactants location will be highlighted. The behavior of leucine and phenylalanine with different partition coefficient will be evaluated.
towards the formation of fructose-leucine and fructose-phenylalanine in aqueous solution and in microemulsion model systems with glyceryl trioctanoate and Tween 20.

In Chapter 6 the main achievements will be summarized reporting which goals have been met and which are the future perspectives of the MR mitigation strategies.
## Chapter 2

### Reactants encapsulation and Maillard reaction

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Encapsulated Reactants</th>
<th>Intermediates</th>
<th>Controlled End Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3-Deoxyglucosone</td>
<td>HMF</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td>Fructofuranosyl cation</td>
<td>HMF</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>Ascorbic acid</td>
<td>Fructosyl-lysine</td>
<td>CML</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>3-MCPD monoester</td>
<td>3-MCPD</td>
</tr>
</tbody>
</table>

*Antonio Dario Troise and Vincenzo Fogliano*

*Trends in Food Science & Technology* 33.1 (2013): 63-74
In the last decades many efforts have been addressed to the control of Maillard Reaction products in different foods with the aim to promote the formation of compounds having the desired color and flavor and to reduce the concentration of several potential toxic molecules. Encapsulation, already applied in food industry for different purposes, can be used as a strategy to get the controlled release of some compounds promoting the Maillard Reaction development in order to mitigate the formation of some undesired compounds. In this review the underneath reaction mechanism, the activity of various reactants, the encapsulation strategies and some possible applications in food processing were discussed highlighting the potentialities of encapsulated ingredients in the modulation of Maillard Reaction.

**Keywords:** Encapsulation, process contaminants, thermal treatments, mitigation strategies
Introduction

The Maillard Reaction (MR) is strictly linked to food quality and safety of thermally treated foods \(^{22, \ 23}\) and along with caramelization and ascorbic acid oxidation, it is the main driver of non-enzymatic browning.\(^5\) MR occurs between a carbonyl compound, mainly the carbonyl moiety of a reducing sugar and an amino group counterpart, which can be an amine, an amino acid, a peptide or a protein.\(^24\) From the chemical point of view, MR can be divided into three main steps. In the early stage of MR, the carbonyl attachments and the formation of a glycosylamine are followed by the formation of two more stable intermediates: 1-amino-1-deoxy-2-ketose the Amadori rearrangement product (ARP) and 2-amino-2-deoxyaldose Heyns rearrangement products (HRP).\(^3\) Various mechanism and a huge variety of pathway have been proposed\(^{25-27}\) The Amadori product fragmentation depends on the pH and on the available reactants and it represented the crucial point for the formation of hydroxymethylfurfural (HMF), acrylamide, furosine, Ne-(Carboxymethyl)-L-lysine (CML) and others MR products (MRPs). Some of these products are desired because of their sensorial and physiological properties while others are not, because they posed a potentially hazard for consumers health.\(^28\) In particular, the decarboxylated Amadori product of asparagine with reducing sugars is the key precursor of acrylamide,\(^{29, \ 30}\) instead both glucose and fructofuranosyl cation can generate HMF through the elimination of two and three molecules of water, respectively.\(^31\) Glyoxal and glycoaldehyde are immediate precursors to the formation of CML, Ne-(Carboxyethyl)-L-lysine (CEL) furosine via Schiff’s base adduct, along with ARP via oxidative cleavage.\(^32, \ 33\) This mechanism was summarized by Namiki and Hayashi\(^{34}\) and by Hofmann and co-workers highlighting the importance of radical intermediates in the early stage of the MR.\(^35\) These species were identified as pyrazinium radical cations, which appear to be key precursors in subsequent MR pathways.

The accurate control of MR in the different foods is often the turning point to obtain products of superior quality. For this reason the optimization of the desired compounds and the reduction of the potentially harmful ones is among the hot topics in the applied food science research. Even if the relationship between MR and encapsulation has not been deepen explored yet, various findings highlights the great potential of encapsulation as mitigation strategy in MRPs formation\(^\)”. Few studies previously published demonstrated that well designed capsules tailored considering processing of the specific foods were able to achieve the desired effect.
**MR and mitigation strategies**

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. In this paper, a bird view discussion about the existing approaches, which are summarized in Figure 2.1, will be the starting point for the evaluation of the encapsulation as an innovative way to reduce the formation of MRPs.

![Figure 2.1: Visual summary of the mitigation strategies available for reducing the formation of undesired Maillard Reaction compounds in foods. The four parts of the circle represented the categories of mitigation strategy developed up to now. In the external parts there is a list of different treatments that was explored for each strategy.](image)

In the last ten years, the scientific efforts have been mainly addressed to the reduction of acrylamide not only in potato and cereal products that are major contributors, but also in coffee, roasted nuts and infant food (CIAA, last updates June 2012). The mitigation steps for acrylamide formation included changes in recipes and formulations such as selection of potato and cereal varieties low in acrylamide precursors; addition of proteins, glycine, cysteine and other amino acids which act as competitors towards asparagine; addition of organic acids, acidulants and calcium ions; addition of the enzyme asparaginase which proved to be one of the most promising tools to control acrylamide content particularly in bakery leavened products.

On the other hand, it has been showed that the above mentioned strategies can often lead to an increased concentration of some undesired compounds such as HMF or 3-monochloropropandiol.
The addition of bivalent cations, such as Ca$^{2+}$, switches the reaction pathway to the dehydration and pyrolysis of glucose leading to an increased formation of HMF and furfural$^{42}$; glycine addition caused the acceleration of MR and the addition of consumable acids favors the rise up of pH.$^{46}$ Prolonging yeast fermentation can efficiently reduce acrylamide concentration in bread, but it is also associated to an increase in the concentrations of 3-MCPD.$^{47-49}$ The use of antioxidants has been also proposed to mitigate the formation of MRPs and in some cases the presence of polyphenols could influence the overall MR development.$^{50}$ Following the reaction mechanism proposed by Namiki and Hayashi and the subsequent studies in taste-active MRPs development,$^{51}$ the effect of phenolic compounds on pyrazinium radical cation formation and on aroma generation has been intensively studied.$^{19,52}$ It was shown that some phenolic compounds, such as (−)-epicatechin, rutin, hydroxycinnamic acid, curcumin behave as trapping agent of carbonyl limiting carbohydrates reactivity under such conditions.$^{19,53,54}$ However, in many cases antioxidants were not effective or even promote the formation of acrylamide.$^{55,56}$

The use of asparaginase to prevent acrylamide formation was probably the most successful example of the enzymatic approach to MRPs mitigation. However, also the use of other enzymes has been proposed. Amadoriase,$^{57-59}$ fructosamine-3-kinase, and the system constituted by fructoselysine-6-kinase plus fructoselysine-6-P deglycase, and FN3K related proteins$^{60}$ are potential smart tools to control the overall extent of MR. Amadoriase acted in the initial steps of MR, the oxidative deglycation of low molecular weight fructosamine or ARP producing deoxyglucosone and amino acids. It has been recently demonstrated that it is able to reduce the formation of CML and protein-bound hydroxymethylfurfural (b-HMF) in a commercial UHT low lactose milk and a β-lactoglobulin model system.$^{61}$

Another mitigation strategies of MRPs formation is to reduce the overall thermal input in order to control the final concentration of heat induced toxicants.$^{62}$ For this purpose several technological strategies have been studied:$^{11}$ ohmic heating,$^{63-65}$ radio-frequencies,$^{66}$ non thermal technologies such as high hydrostatic pressure,$^{67}$ pulsed electric field.$^{68}$ The relationship between MRPs formation and high pressure remains still unclear, in fact some studies verified the acceleration of early Maillard reaction pathways under high pressure, e.g. reaction products formed from tryptophan and glucose or xylose, and the slowdown of subsequent reaction steps.$^{69}$ Others authors reported that the formation and subsequent degradation of ARP, along with the dicarbonyl cross-linking formation, was accelerated by high pressure.$^{70-72}$ Pulsed electric field
technology represents an effective tool in the reduction of HMF formation.\(^7^3\) Interestingly, Anese and Quarta highlighted the fact that the vacuum treatments allowed removal of HMF and furfural from previously hydrated coffee powder, however they observed a significant decrease in total volatiles compounds that are fundamental for aroma formation.\(^7^4\)

Kinetic modeling of MRPs formation in the various food industrial processes represented one of the most important emerging issues.\(^1^3,7^5,7^6\) The main goal here is to produce kinetic models that would allow a design of food process minimizing the formation of negative compounds keeping the desirable sensorial properties.

**Encapsulation: current use in foods**

Encapsulation has been described as “the technology of packaging solid, liquid and gaseous material in small capsules that release their contents at controlled rates over prolonged periods of time”.\(^7^7,7^8\) Therefore, encapsulation technologies may play a key role in the stabilization and protection of sensitive nutrients and food ingredients.\(^7^9\)

Several techniques have been currently used for encapsulation such as spray drying, spray chilling or spray cooling, extrusion coating, fluidized bed coating, liposome entrapment, coacervation, inclusion complexation, centrifugal extrusion and rotational suspension separation.\(^8^0\) Other approaches involve nanoemulsions and nanodispersion that can be used to incorporate and deliver a broad range of molecules. Among this category different techniques are present: nanodispersions, nanocapsules, nanostructured multilayer emulsions, nanostructured multiple emulsions, nanolaminates and biopolymeric stucures.\(^8^1\) Moreover the relationship between MR and nanoemulsion based system has previously been explored by Garti and co-workers for aroma formation and control.\(^8^2,8^3\)

The process has three main objectives: formation of a wall around the bioactive compound, avoiding leakages, ensuring that undesired materials are kept out. Encapsulation allowed the protection of the core material from adverse environmental, physical, chemical and mechanical stimuli. The immediate goals of encapsulation in food are to increase the shelf life of the product, and to promote a controlled release of the core material.\(^8^4\) The prominent reasons for encapsulation are: (a) protection of the core materials from degradation by reducing its reactivity toward the food matrix and the environment; (b) reduction of the transfer rate of the core
material to the environment; (c) improvement of the physical characteristics of the original material; (d) tailoring the release of the core material over time; (e) masking undesired flavors or tastes of the core material; (f) uniform dispersion in the host material, when only small amounts are required; (g) helping the separation of reactive molecules in the mixture.\textsuperscript{85, 78} This last point is exactly the one which has been considered to apply encapsulation as a new strategy in the reduction of MRPs formation. The approach, based on the controlled release of a reactants during the process time, will be detailed in the following paragraph.

In Figure 2.2 the main bioactive molecules that can be encapsulated and delivered into foods are summarized. They can be divided in two macro-categories: lipophilic bioactive molecules and hydrophilic bioactive molecules. In the first group there are polyunsaturated fatty acids (PUFA), carotenoids, tocopherols, flavonoids, polyphenols and phytosterols.\textsuperscript{86}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hydrophilic_and_lipophilic_molecules.png}
\caption{HYDROPHILIC MOLECULES | LIPOPHILIC MOLECULES}
\end{figure}

\textbf{Figure 2.2:} Compounds of nutritional interest which can be potential target of the encapsulation strategies are shown divided in two macro-categories: hydrophilic bioactive molecules and lipophilic bioactive molecules. In the first group naringenin, ascorbic acid, tyrosol, 3-hydroxytyrosol and various cations are shown. In the second group there are polyunsaturated fatty acids (PUFA), carotenoids, tocopherols and phytosterols.\textsuperscript{86}

In the last decades many efforts have been dedicated to the encapsulation of lipophilic bioactive molecules, particularly PUFA.\textsuperscript{87} These compounds cannot be easily dissolved in food products therefore encapsulation is used to enhance solubility and to prevent oxidation and peroxidation in several food matrices.\textsuperscript{88, 89} Many encapsulation approaches have been proposed for including also
other lipids in a large variety of food products.\textsuperscript{86} Maltodextrin and spray coating were found to be useful in protecting carotenoids and anthocyanins against isomerization and oxidation\textsuperscript{90,91} several techniques could be efficiently applied to phenols encapsulation,\textsuperscript{92} such as spray drying in maltodextrin or chitosan\textsuperscript{93} coacervation in alginate and calcium alginate; \textsuperscript{94} liposome through thin film evaporation\textsuperscript{95} and inclusion with a huge variety of coating material.\textsuperscript{85}

Phenolic compounds show some features at the interface between hydrophilic and lipophilic properties, so the choice of the wall material is a crucial point. In a recent work, Vitaglione et al.\textsuperscript{96} tested the human bioavailability of cocoa flavanols in a gastric resistant high amylose maize starch showing that encapsulated ingredient increased flavanol delivering into the gut decreasing their intestinal absorption. Similar results were obtained for bread with double coating (cellulose and hydrogenated vegetables oil) encapsulated curcumin.\textsuperscript{97}

On the opposite side the encapsulation of water soluble molecules, such as proteins, peptides, amino acids, bioactive carbohydrates and essential minerals, required different coating features.\textsuperscript{86} Overlooking the functional properties of these molecules, the main challenges involve the capabilities of the wall material in releasing the bioactive components in the site of action and the capabilities in protecting the molecules from the production process\textsuperscript{98} avoid adverse effects in texture, appearance and flavor.\textsuperscript{99,100} For the incorporation of essential minerals the protection from the environment, the limitation of their catalyzing activity, the increasing of their low bioavailability and the control of their physical and chemical activity are the most prominent challenges.\textsuperscript{101}

Encapsulation has many application in the sensory area: it might be used to block some undesired molecules inside the wall material, preventing the reaction between the chemical compounds and the so-called gate keeper proteins of the taste-buds.\textsuperscript{102} For this purpose, the most common way is to encapsulate the food component linked to the bitter taste inside ciclodextrins; in particular \(\beta\)-cyclodextrin have been efficiently used to mask the astringent taste of aliphatic carbonyl compounds in soybeans\textsuperscript{103} the sweet taste of glycyrrhizin; the bitter aftertaste of stevioside, rubusoside, limonin and naringin;\textsuperscript{104} elimination of unpleasant taste of fish oil and stabilization against oxidation of PUFA.\textsuperscript{105}

Also encapsulation has found some emerging applications in the manufacturing of healthy foods: bioactive food peptides and casein phosphopeptides have received special attention in particular
for antihypertensive, immune-stimulatory and ACE inhibition, mineral binding, cancer prevention activity both in milk product and in cheese or yoghurt. The encapsulation not only is particularly useful for the delivery of several kinds of peptide, for example it protects the peptides from the hydrolysis, but also for the reduction or elimination of bitter taste. As observed earlier the utilization of protein hydrolysates in food systems is frequently hindered due to their bitterness, hygroscopicity and reactivity.

**Modulating Maillard reaction through encapsulation**

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 was 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 **Figure 2.3**.

**Figure 2.3:** Possible encapsulation strategies to control Maillard Reaction development. Undesired Maillard reaction products which can be controlled using encapsulation strategies were highlighted into blue circles. The key compounds that can be encapsulated were highlighted into red circles. R, R1 and R2 can represent both PUFA both saturated fatty acids. R3 and R4 are other amino acids.
Chapter 2

**Sodium Chloride**

The effect of salt has been extensively investigated during the last decades. Gökmen and Senyuva tested the effect of several monovalent, divalent, and trivalent cations on MRPs formation.\(^{42}\) Generally monovalent and bivalent cations favor 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.\(^{31, 42}\) This effect have been widely demonstrated both in commercial dextrose preparations, in cookie model systems in the presence different concentration of NaCl, in partial dehydrated cherry tomato and in grapefruit, respectively.\(^{110-112}\)

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.\(^{113, 114}\) 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.\(^{49}\) In addition, amino acid hydrochloride salts have greater ability to chlorinate glycerol than a mixture of sodium chloride and amino acids.\(^{114}\) 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.\(^{114}\)

The encapsulation of sodium chloride is an effective strategy to control the formation of some MRPs. Fiore and co-workers 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.\(^{115}\) 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 do 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. 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.

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 anemia and to rise up the poor bioavailability of the iron naturally present in milk.\textsuperscript{116} Besides these 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\textsuperscript{117} or to complexes able to catalyze browning.\textsuperscript{118, 119} However, in some cases, metal ions are able to suppress browning\textsuperscript{9} or can be effectively used to catalyze the precipitation and the successive removal of the brown material.\textsuperscript{120} Ames and Fallico 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.\textsuperscript{121} Kwak and Lim 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.\textsuperscript{122} They confirmed the results previously obtained by Morita and Kashimura demonstrating the oxidative effect of transition metals ions in browning enhancement.\textsuperscript{123} Ramonaityte and co-workers 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 non-dialyzable fractions of the system verifying that Fe, Cu and Zn ions have a clear impact on colored compounds formation.\textsuperscript{124}

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 capsules wall 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 showed that Fe\textsuperscript{2+} could be trapped inside the inner water droplets and released at a controlled rate in a model system.\textsuperscript{101} 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, 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.

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.

**Ascorbic acid**

In milk infant formulas iron supplementation is often associated with ascorbic acid salt for nutritional purposes. The iron-ascorbate system had deleterious action toward protein and amino acids in milk formula promoting their degradation and polymerization. Ascorbic acid acts as a pro-oxidant in presence of iron or other transition metals that, along with the production of hydrogen peroxide, catalyze the oxidation of ascorbic acid into dehydroascorbate. This reaction implies various modifications in the food, that were first evaluated by Ueda et al. in a food model system, then by Leclère and Birlouez-Aragon 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 blockade. CML has been widely used as marker of protein carboxylation not only in food, but also in human tissue and urine. Interestingly, Dunn and co-workers 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 antioxidative 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’s group. 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, in particular the authors stated that it is not recommended to fortify canned and jarred vegetables with vitamin C before thermal treatment.

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.\textsuperscript{136} 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.\textsuperscript{137-140} The loading of ascorbic acid in chitosan nanoparticles prepared by ionic gelation was also reported.\textsuperscript{141} There is a huge variety of food where these encapsulated ingredients can be applied however the key 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 kind of products.

\textit{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 flavor formation thus improving sensorial acceptability.\textsuperscript{96} There are many factors to be considered in the evaluation of encapsulated lipid stability which have been summarized by Decker and McClements’ group.\textsuperscript{142} From MR point 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.\textsuperscript{143-145} 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.\textsuperscript{142, 146, 147}

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 reaction is the irreversible formation of early stable intermediates such as the Amadori product and the lipid hydroperoxides for MR and lipid oxidation respectively.\textsuperscript{148} Lipid oxidation products influence directly MR, producing several molecules that differ from the ones produced in absence of lipids.\textsuperscript{148} Gökmen and co-workers observed that incorporating encapsulated PUFA in bread resulted in a decrease of HMF and acrylamide.\textsuperscript{149} They explained these results considering that carbonyls arising from the thermoxidation of PUFA during baking can promote the conversion of asparagine into
The possibility of using encapsulation to modulate a chemical reaction, specifically the MR, opened several fields of applications and many different food products could benefit by the use of tailored encapsulated ingredients. Few cases have already been explored such as the use of encapsulated sodium chloride in bakery products or brine; as well as the use of encapsulated 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. The interrelation between lipid oxidation and MR is evident in the formation of acrylamide and several papers deal with the mechanism of formation: via acrolein which provide the reactive carbonyl moiety, via the reaction of lipid derivatives and asparagine, and via the influence of lipid oxidation in various fat-rich model systems. 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. 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.

**Conclusion and future perspectives**

The mitigation of potentially harmful compounds formation is one of the main target of food industries and, as showed above, the encapsulation of bioactive molecules or reactants offers several opportunities in this respect. In the last years many papers on encapsulated ingredients focused on the physical and chemical properties of core and wall materials have been published. They linked the characteristics of the ingredients to the food matrix and to the biological outcomes, such as bioavailability, sensorial masking and gut delivery.

The possibility of using encapsulation to modulate a chemical reaction, specifically the MR, opened several fields of applications and many different food products could benefit by the use of tailored encapsulated ingredients. Few cases have been already explored such as the use of encapsulated sodium chloride in bakery products or brine; as well as the use of encapsulated acrylamide. When the oil is encapsulated, reactive carbonyls are not available for this reaction due to prevention of its thermoxidation during baking.

In a meat model-system consisting in various amino acids, sugars and phospholipids, Elmore and co-workers 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. The interrelation between lipid oxidation and MR is evident in the formation of acrylamide and several papers deal with the mechanism of formation: via acrolein which provide the reactive carbonyl moiety, via the reaction of lipid derivatives and asparagine, and via the influence of lipid oxidation in various fat-rich model systems. 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. 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.

**Conclusion and future perspectives**

The mitigation of potentially harmful compounds formation is one of the main target of food industries and, as showed above, the encapsulation of bioactive molecules or reactants offers several opportunities in this respect. In the last years many papers on encapsulated ingredients focused on the physical and chemical properties of core and wall materials have been published. They linked the characteristics of the ingredients to the food matrix and to the biological outcomes, such as bioavailability, sensorial masking and gut delivery.

The possibility of using encapsulation to modulate a chemical reaction, specifically the MR, opened several fields of applications and many different food products could benefit by the use of tailored encapsulated ingredients. Few cases have been already explored such as the use of encapsulated sodium chloride in bakery products or brine; as well as the use of encapsulated
The possibility of using encapsulation to modulate a chemical reaction, specifically the MR, outcomes, such as bioavailability, sensorial masking and gut delivery.

They linked the characteristics of the ingredients to the food matrix and to the biological focused on the physical and chemical properties of core and wall materials have been published. Several opportunities in this respect. In the last years many papers on encapsulated ingredients opened several fields of applications and many different food products could benefit by the use of tailored encapsulated ingredients. Few cases have been already explored such as the use of EPA and DHA.

Products which are easily formed particularly form long chain polyunsaturated fatty acids such as co-workers observed that phospholipids interact with sugars and amino acids, not only with the derived volatiles, such as aldehydes, alcohols and alkylfurans in many foods.

Ascorbic acid; iron in infant formula or other functional beverages. However, encapsulation strategies might be successfully employed also in other significant source of MRPs such as cheese, chocolate-flavored drink, salad dressing and sauces. The above mentioned food matrix would be also suitable to investigate other aspects of encapsulation and MR such the formation of desired compounds in particular of those contributing to the aroma. The presence of encapsulated reactants could easily switches the reaction pathways and offer the possibility to modulate the concentration of desired molecules. On the opposite the formation of these compounds could be influenced negatively in presence of coating material, i.e. the presence of waxy material or lipidic coating can alter the aroma.

Despite the exciting potentiality there are several gaps limiting the large use of this technology in the current food production. Not all the capsules are resistant to the process conditions: temperature and pressure can cause the disruption of the coating and process-resistant capsules might be no more suitable for the intended purposes. Moreover, in some cases a negative sensorial impact was reported for encapsulated ingredients particularly an increase of granularity in creams and beverages.

Finally, the cost in use of the encapsulated products might be not always sustainable for mass production and should be evaluated case by case. This latter aspect is also strictly associated with legislative issue: in some cases encapsulated ingredients can be considered novel foods and this would require specific authorization based on risk assessment.
Chapter 3

Faox enzymes inhibit development of the Maillard Reaction in storage both in the of protein glucose model system and low lactose UHT milk.

Antonio Dario Troise, Nina A. Dathan, Alberto Fiore, Giovanni Roviello, Anna di Fiore, Simonetta Caira, Marina Cuollo, Giuseppina De Simone, Vincenzo Fogliano and Simona M. Monti

Amino acids 46.2 (2014): 279-288
Chapter 3

Faox enzymes inhibit development of the Maillard Reaction in storage both in the of protein glucose model system and low lactose UHT milk

Antonio Dario Troise, Nina A. Dathan, Alberto Fiore, Giovanni Roviello, Anna di Fiore, Simonetta Caira, Marina Cuollo, Giuseppina De Simone, Vincenzo Fogliano and Simona M. Monti

Amino acids 46.2 (2014): 279-288
Fructosamines, also known as Amadori products, are formed by the condensation of glucose with the amino group of amino acids or proteins. These compounds are precursors of advanced glycation end products (AGEs) that can be formed either endogenously during aging and diabetes, and exogenously in heat-processed food. The negative effects of dietary AGEs on human health as well as their negative impact on the quality of dairy products have been widely described, therefore specific tools able to prevent the formation of glycation products are needed. Two fructosamine oxidase enzymes isolated from *Aspergillus sp.* namely, Faox I and Faox II catalyze the oxidative deglycation of Amadori products representing a potential tool for inhibiting the Maillard reaction in dairy products. In this paper, the ability of recombinant Faox I and II in limiting the formation of Ne-(Carboxymethyl)-L-lysine (CML) and protein-bound hydroxymethylfurfural (b-HMF) in a commercial UHT low lactose milk and a β-lactoglobulin (β-LG) glucose model system was investigated. Results showed a consistent reduction of CML and b-HMF under all conditions. Faox effects were particularly evident on b-HMF formation in low lactose commercial milk. Peptide analysis of the β-LG glucose system identified some peptides, derived from cyanogen bromide hydrolysis, as suitable candidates to monitor Faox action in milk-based products. All in all data suggested that non-enzymatic reactions in dairy products might be strongly reduced by implementing Faox enzymes.

**Keywords:** Faox, Maillard reaction, CML, b-HMF, AGEs, milk
Introduction

Initial steps of Maillard reaction (MR) involved reversible reactions between carbohydrates or lipids carbonyl moiety and amines to form a Schiff base which undergoes rearrangements producing the Amadori product (AP). Stable covalent adducts or cross-links called advanced glycation end products (AGEs) are formed by dehydration and fragmentation both in human body and in foods (dietary AGES or Maillard reaction end products). It has been hypothesized that dietary AGES represent a consistent risk factor, due to their ability to induce oxidative stress and inflammation by binding to cell surface receptors, thus posing severe risk in particular for some categories. The formation of dietary AGES in dairy products largely occurring under severe heat treatments, should be always prevented for nutritional and sensorial reasons.

Several tools have been developed to limit glycation and thus the formation of dietary AGES either by chemical approaches, using carbonyl traps, such as rutin, natural antioxidants, sugar autoxidation inhibition, reactant encapsulation technology and bivalent cation addition, or by enzymatic approaches employing enzymes, such as fructosamine oxidase fructosamine-3-kinase, and FN3K-related proteins. Two fructosamine oxidase enzymes, hereafter referred to as Faox I and Faox II, which have been isolated by Monnier and co-workers from fungi, capable of using the Amadori products as substrates, catalyzed the oxidative deglycation of low molecular weight fructosamine or Amadori product to yield deoxyglucosone and amino acids. After the catalytic cycle, reduced FAD is oxidized by molecular oxygen with the concomitant release of one molecule of hydrogen peroxide. Faox I and II have been reported to react differentially with AP on free amino acids and AP formed on small peptides. In contrast, glycated lysine residues bound to globular proteins, such as bovine serum albumin (BSA) are poor substrates for the enzyme.

The reason for the differential activity towards variably sized glycated substrates was explained by the resolution of Faox X-ray structure by Monnier and co-workers. Faox II is a two domain FAD-enzyme with an overall topology similar to that of monomeric sarcosine oxidase, where the catalytic site is buried in a 12 Å deep pocket which is not easily accessible to glycated globular proteins. It has been shown that when Faox I was added during the glycation reaction of small globular proteins, such as insulin or beta-lactoglobulin (β-LG), the development of MR was significantly reduced. Although any clear mechanism was not identified to explain this effect, it has been hypothesized that it was due to a temporary conformational changes occurring during glycation making the AP accessible to the enzyme catalytic site. In this paper, we aimed at
clarifying this point, investigating the capacity of Faox I and Faox II to reduce AGEs formation when added during the glycation of milk and globular proteins. The effects of Faox I and II were evaluated using both a UHT low lactose commercial milk (LLM) and a β-LG-glucose model system. Glycated peptides as well as non-enzymatic glycation markers such as Ne-(Carboxymethyl)lysine (CML) and bound 5-hydroxymethylfurfural (b-HMF) were quantified while glycated b-LG peptides were characterized by MALDI-TOF analysis. The results showed a reduction in glycated peptides CML and b-HMF in all systems investigated.

Materials and methods

Expression and purification of Faox I and II

Both Faox I and II cDNAs were received as a kind gift from Prof. Monnier. They were both PCR amplified and cloned in the AgeI-HindIII site and AgeI-Xhol site respectively of pET28-SUMO3GFP (a kind gift from EMBL, Heidelberg) using site-specific primers: Faox I: 5’-TCATCTACCTCTGCTCTCTCCAATC-3’ and 5’-GTCTCGAAGCTTTTATCACGGACCTCTGCTCTCTCCAATC-3’; FaoxII: 5’- TCATCTACCGGTGGCATGGCGGTAACCAAGTCATCTTC-3’ and 5’-CGGCGGCCGCTCGAGTTATCATACTTGGAAATATCTCTATATTGTAC ATTTGTCC. Both recombinant Faox I and II constructs were expressed in BL21(DE3) cells (Novagen) 16h at 20 °C in the presence of 0.1mM IPTG, yielding soluble protein. Faox I was lysed in: 20 mM Tris (pH 8.0), 10mM Imidazole, 250 mM NaCl, 0.1% Triton X-100, in the presence of PMSF, DNaseI and lysozyme, clarified and purified by FPLC, using an ÄKTA system with a 1ml HisTrapFF column (GE Healthcare) by stepwise elution. The protein eluted > 90% pure in 110 mM Imidazole, 20 mM Tris (pH 8.0), 250 mM NaCl and the fusion protein was removed with SenP2 protease (prepared in-house from pETM11SenP2 a kind gift from EMBL, Heidelberg) at a dilution 1:800, 16h at 20 °C. The cleaved protein was concentrated on Amicon 10 KDa MWCO to 10 mg/ml and polished on a size exclusion column, Sephadex 75. Faox II lysate was prepared as Faox I, but at pH 8.5 and in the initial purification step the recombinant protein eluted at 250 mM imidazole. The fusion protein was cleaved as before, concentrated, with a change of buffer (20 mM Tris, 50 mM NaCl, pH 8.5) on Amicon 10 KDa MWCO and isolated from the SUMO/6xHis Tag on a 1ml MonoQ column, eluting cleanly using a linear salt gradient.
**Enzymatic Activity**

The activity of recombinant Faox I and Faox II was evaluated following the production of $\text{H}_2\text{O}_2$ in a peroxidase-coupling reaction at $25^\circ \text{C}$ by monitoring the formation of a quinone dye at 505 nm ($\varepsilon$, 7210 mol$^{-1}$ L cm$^{-1}$). The reaction mixture contained 10 mM potassium phosphate pH 7.0, 0.38 mM aminoantipyrine, 0.6 U horseradish peroxidase, 0.5 mM phenol, in a final buffer volume of 1 ml. Synthetic $\varepsilon$-Fructose-lysine was used as substrate. One unit of enzyme activity was defined as the amount of enzyme that produced 1 $\mu$mol of quinine dye per minute.$^{275}$

**Circular Dichroism**

All CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier temperature control system [Model PTC-423-S]. Molar ellipticity per mean residue, $\theta$ in deg cm$^2$/dmol, was calculated from the equation: 

$$[\theta] = \frac{[\theta]_\text{obs} \times \text{mrw}}{10 \times I \times C},$$

where $[\theta]_\text{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, C is the protein concentration in mg/mL, and I is the optical path length of the cell in cm. Far-UV measurements (183–250 nm) were carried out at 20 $^\circ$C, at time constant of 4 s, 2 nm band width, scan rate of 10 nm/min, using a 0.1 cm optical path length cell and a protein concentration of 0.2 mg/mL in 6.6 mM buffer phosphate pH 8.0. CD spectra were signal averaged over at least three scans, and baseline was corrected by subtracting a buffer spectrum. CD of Faox II at different pH were performed after dialysis in different buffers such as 10 mM Tris, 10 mM NaCl, pH 9.0; 15 mM potassium phosphate, 10 mM NaCl, pH 6.0; 15 mM NaAc, 10 mM NaCl, pH 5.2; 15 mM NaAc, 10 mM NaCl, pH 4.0. Before registering the CD spectra, dialyzed samples were diluted in water. Thermal unfolding curves were determined by recording the molar ellipticity at 222 nm, using a scanning rate of 1$^\circ$C/min ranging from 20 to 90$^\circ$C.

**Multiple-Angle Light-Scattering (MALS) analysis**

The monomeric form of recombinant Faox I was determined by combining size exclusion chromatography (SEC) with a multiple-angle light-scattering (MALS) instrument. Experiments were run at 0.5 ml/min in 20 mM Tris, 150 mM NaCl, pH 7.0 buffer loading 300 $\mu$g enzyme on a Superdex 10/30 size exclusion column (GE Healthcare) connected to an FPLC ÄKTA purifier system which in turn was connected to a Refractive index (Shodex RI 101) followed by a Mini Dawn Treos (Wyatt Technology, USA) Light Scattering instrument. The online measurement of the intensity of
the Rayleigh scattering as a function of the angle as well as the differential refractive index of the eluting peak in SEC was used to determine the weight-average molecular mass (Mw) eluted proteins, using the Astra 5.3.4.14 software (Wyatt Technologies).\textsuperscript{176}

**Experimental systems**

The experiments were performed both on a commercial low lactose UHT milk and on a β-LG glucose model system. All samples were prepared under a sterile hood to avoid bacterial contamination, being filtered before use through 0.45 μm (Faox I and Faox II) or 0.22 μm cutoff (β-LG and glucose) membranes. Briefly, 10 mg/ml β-lactoglobulin (Sigma) was added to glucose (0.5M) in 200 mM phosphate buffer at pH 7.2 with a final volume of 1 ml. UHT low lactose commercial milk (LLM) was not filtered before usage in order to leave its physicochemical properties unaltered, and enzymes were added at a protein ratio of 1:1000. After Faox enzymes addition, samples were stored at 37 °C for 17 days. When the enzymes were not mixed to samples, the equivalent volume of buffer was added. Following incubation, samples were frozen at -20°C before analysis.

**Evaluation of Maillard Reaction development in the model system**

The MR development was assessed by measuring b-HMF and CML. Glycation of specific peptides of the protein-glucose system was evaluated by MALDI TOF MS analysis.

**Quantification of b-HMF**

The extraction procedure was performed according to Morales and Jiménez-Pérez with slight modifications:\textsuperscript{177} 500 μl of low lactose commercial UHT milk or β-LG glucose model system were transferred to a 3 kDa regenerated cellulose centrifugal filter unit (Amicon Ultra, Millipore, Ireland) and centrifuged three times at 4200 rpm for 30 min; 200 μl distilled water was added following each cycle. The final volume of the protein concentrate was adjusted to 1ml; 500 μl were mixed with an equal volume of 0.3 N oxalic acid in an plastic tube tightly closed to prevent evaporation. The tube was heated in a water-bath system at 100 °C for 60 min. After cooling to room temperature, 1 ml of 40% (w/v) trichloroacetic acid (TCA) solution was added. The mixture was stirred for 5 min and centrifuged 15 min at 14800 rpm. The supernatant was passed through a 0.45 μm regenerated cellulose filter and injected onto an UPLC system that consisted of two LC-
20AD class VP pumps and a SPD-20A UV-Vis detector equipped with a SIL-20A autosampler, 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 Hydro-RP column 80Å, 250 x 4.6 mm 4 µm (Phenomenex, Torrance, CA) was used for the chromatographic separation. The UV-Vis 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 determination $r^2$ was 1 after three replicates. The limit of detection (LOD) was 0.050 µg/mL, whereas the limit of quantification (LOQ) was 0.150 µg/mL. All of the analyses were performed in quadruplicate by injecting 20 µL of milk or model system extracts in the system and the results expressed as µg per ml sample.

Quantification of Ne-(Carboxymethyl)-L-lysine (CML)

The analysis of CML was performed according to Delatour and co-workers and Fenaille and co-workers (2006) with slight modifications. Briefly, 60 µL of LLM (corresponding to 1.92 mg of protein) or 192 µL of β-LG glucose model system (corresponding to 1.92 mg of β-LG) were diluted in 450 µL of 0.2 M sodium borate (pH 9.2). An aliquot of 500 µL of sodium borohydride 1.0 M in 0.1 M NaOH was added in the mixture and the solution was incubated overnight at room temperature in order to achieve the complete reduction of Amadori compounds. Subsequently 1 ml of TCA was added to the mixture and the samples were centrifuged for 10 min at 4000 rpm (4 °C). After careful removal of the supernatant, the protein pellet was diluted in 2 ml of 6 N HCl . The mixture was incubated for 24 h at 110°C in an air forced circulating oven and 1 ml was evaporated under a gentle flow of nitrogen. The samples were reconstituted in 990 µL of water and 10 µL of the internal standard d2-CML were added in order to obtain a final concentration of 238 ng/mL. Samples were loaded onto equilibrated Oasis HLB 1cc cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described, finally 20 µl were injected onto the LC-MS/MS system. Identification and quantification of CML and d2-CML were performed on API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA) coupled to a Turboionspray (TIS) interface, equipped with an HPLC binary micropump series 200 (Perkin-Elmer, Waltham, MA). CML and d2-CML separation was achieved on a reversed – phase HPLC column (TSKgel-amide 80, 2.0 mm x 25 cm, Tosoh Bioscience, Tokyo, Japan) using the following mobile phases: A, 0.1% formic acid and B, acetonitrile. Compounds were eluted at 200 µL/min through the following gradient of solvent B (t in [min]/[%B]): (0/10), (4/10), (8/90), (10/10), (12/10). With the
above described chromatographic conditions, typical retention times of CML and d2-CML were 3.5 min. Positive electrospary ionization was used for detection and the source parameters were selected as follows: spray voltage: 5.0 kV; capillary temperature: 350 °C, dwell time 100 ms. The chromatographic profile was recorded in multiple reaction monitoring mode and the characteristic transitions were monitored in order to improve selectivity. CML was quantified using a linear calibration curve built with specific solutions of CML and d2-CML dissolved in water (50–1000 ng/mL). The LOD and LOQ were, respectively, 10 and 30 ng/mL for CML, and the coefficient of determination $r^2$ was 0.9998. The internal standard was used for the recovery test, varying 70 to 85%. All of the analyses were performed in quadruplicate and the results expressed as ng per milligram sample.

*Evaluation of protein glycation on the β-LG-glucose model system*

*Reduction and carboxymethylation procedure*

Purified β-LG (5 mg) was dissolved in 300 µl of 0.3 M Tris–HCl, pH 8.0, containing 6 M -guanidine–HCl, 1 mM EDTA, and treated with dithiothreitol (10 : 1 molar excess with respect to cysteine residues) at 37 °C for 2 h. Carboxymethylation was carried out with a five-fold molar excess of iodoacetic acid with respect to dithiothreitol, at pH 8.0, at room temperature for 30 min in the dark. The sample was desalted by gel filtration through a PD-10 G-25 column (Bio-Rad) in 50 mM ammonium bicarbonate, pH 8.5, and freeze-dried.

*Protein hydrolysis*

Cyanogen bromide (CNBr) hydrolysis of β-LG was performed in 70% (w/w) trifluoroacetic acid at room temperature overnight using a ratio of 40 mol of CNBr per mole of methionine. The volatile side products of the reaction, methyl-thiocyanate, excess cyanogen bromide, and trifluoroacetic acid were removed by freeze-drying.

*MALDI-TOF analysis*

MALDI-TOF MS analyses were performed on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with an N$_2$ laser ($\lambda = 337$ nm). DHB was used as matrix for analyzing peptides resulting from protein hydrolysis. The matrix was prepared by dissolving 10 mg of DHB in 1 mL of aqueous 50% (v/v) acetonitrile containing 1% (v/v) PA. The
instrument operated with an accelerating voltage of 20 kV, a grid voltage of 95% of the accelerating voltage, a guide wire of 0.05% and a delayed ion extraction time of 175 ns, for peptides. External mass calibration was performed with the signal of the matrix dimer at \( [M+H]^+ = 379.05 \) and with the monoisotopic masses of peptide standards, including angiotensin I \( ([M+H]^+ = 1296.68) \) and bovine insulin \( ([M+H]^+ = 5730.61) \), thereby achieving an accuracy in the measurement of the peptide mass better than 80 ppm. The mass spectra were acquired in positive linear ion mode. Raw data were elaborated using the software program Data Explorer version 4.0 (Applied Biosystems). Signals of mass spectra were identified according to the expected molecular mass from the known milk protein sequences, taking into account CNBr specificity according to bioinformatics tools, such as MASCOT 2.3 software (Matrix Science, London, UK) or other on-line resources such as ExPASy FindPept or ProteinProspector.

Statistical analysis

Statistical analyses were performed at least in triplicate and analysis of variance using Duncan's new multiple range test was performed, using XLStat Pro v.11.3 (Addinsoft, New York, NY).

Results and discussion

Biochemical characterization of Faox I and Faox II

pET28SUMO-Faox I and pET28SUMO-Faox II were obtained by cloning PCR-amplified cDNAs in the AgeI– HindIII and Age– Xhol sites, respectively, of pETM28SUMO. The resulting clones were verified by bidirectional sequencing. Bacterial expression and purification allowed us to obtain high yields of proteins with [98 % purity, as assessed by LC–ESI–MS and SDS-PAGE. Enzyme-specific activity using fructose-lysine as substrate was in agreement, as previously described.\(^{57}\) A study of the enzymes in solution by SEC-MALS analysis showed that Faox I and Faox II are monomeric proteins with a MW value of 46,030 ± 46 and 45,570 ± 45 kDa, respectively, with a R<sub>H</sub> of 3.20 ± 0.01 nm. The recombinant enzymes were also characterized in solution by far-UV circular dichroism (CD) spectroscopy. Spectra suggest that native Faox I and Faox II display a high degree of secondary structure with 31 % alpha helix and 23 % beta strand for both enzymes according to the variable selection method (CDSSTR), using DICHROWEB. These data obtained in solution are in agreement with Faox I, whose three-dimensional structure was characterized by X-ray crystallography by Monnier and co-workers.\(^ {57}\) To investigate the pH stability of Faox II, CD spectra at four different
pH: 9.0, 6.0, 5.2, and 4.0 were acquired (Figure 3.1a). Data show that changes in pH do not affect CD spectra of Faox II that preserves almost all of its secondary structure as well as its specific activity towards fructose-lysine substrate as shown in Figure 3.1b). In particular, 82, 86, 55 % of relative-specific activity was preserved at pH 6.0, 5.2 and 4.0, respectively. The melting points at the different pH ranged from 52.0 to 58.8 C after which the enzyme aggregated (data not shown). These events were irreversible under the conditions used, likely due to cross-linking between the six cysteine present within the amino acid sequence. 

\[ \text{Figure 3.1: A, upper panel overlay of CD spectra of Faox II at four different pHs: pH 4.0 (black line), pH 5.2 (blue line), pH 6.0 (green line), pH 9.0 (red line); B, bottom panel, relative specific activity of Faox II at four different pHs: 4.0, 5.2, 6.0, 9.0.} \]

\text{Faox enzymes reduce the formation of CML in low lactose milk (LLM) and protein glucose model system}

The two experimental MR-sensitive systems used in this work were incubated in the presence of Faox enzymes at 37 °C for 17 days. The aim of this prolonged incubation time was to investigate the enzymes ability to limit glycation during product shelf life. At the end of the storage time CML, which is a common and well characterized marker of non-enzymatic glycation was measured.\textsuperscript{13} As
shown in Figure 3.2 in the β-LG-glucose system, the addition of Faox I or Faox II determined a decrease in CML formation of 24 and 38 %, respectively.

A similar set of experiments was also carried out on UHT low lactose commercial milk. In this milk, very reactive carbohydrates, such as glucose and galactose are formed by lactose hydrolysis, thus determining a fast browning and off flavor development\textsuperscript{3, 181} and possibly a decrease of milk nutritional properties.\textsuperscript{182} The CML concentration found in a commercial low lactose milk was 14 ng/mg of protein and this value almost doubled after storage at 37 °C for 17 days. As shown in Figure 3.3, when Faox I and Faox II were added a reduction of CML concentration of 65 and 58 %, respectively, was detected.
It is worth noting that in the conditions applied, the effect of Faox I and Faox II on the CML formation was more evident in low lactose commercial milk than in the protein glucose model system. One possible explanation for this finding might be that part of the milk protein fraction is formed by small peptides and free amino acids that could be suitable substrates for Faox I and Faox II action.

* Faox enzymes reduce the formation of bound hydroxymethylfurfural (b-HMF) in low lactose commercial milk and protein glucose model system

The effect of Faox I and II was also assessed by measuring the levels of b-HMF which is formed during the early stages of the Maillard reaction. b-HMF can be considered as a reliable index of the extent of the MR providing an accurate estimation of glycated protein lysine residues. As shown in Figure 3.4, the addition of Faox enzymes to protein glucose system just before thermal incubation lowered its level by 42 % and 30 %, respectively.
Figure 3.3: CML content in a UHT low lactose commercial milk at time zero (LLM T0) after storage at 37°C for 17 days (LLM). Mean change in CML reduction was significant in relationship to the control after incubation using Duncan’s test (*p < 0.05); n = 4.

It is worth noting that in the conditions applied, the effect of Faox I and Faox II on the CML formation was more evident in low lactose commercial milk than in the protein glucose model system. One possible explanation for this finding might be that part of the milk protein fraction is formed by small peptides and free amino acids that could be suitable substrates for Faox I and Faox II action. Faox enzymes reduce the formation of bound hydroxymethylfurfural (b-HMF) in low lactose commercial milk and protein glucose model system.

Figure 3.4: Bound-HMF content in a milk-like model system after storage at 37°C for 17 days. Mean change in b-HMF reduction was significant in relationship to the control after incubation using Duncan’s test (*p<0.05); n = 4.

Data of Figure 3.5 showed that, as already observed for CML, the effect of Faox enzymes was much more evident in low lactose commercial milk samples than in the β-LG glucose system. Commercial milk at time zero of the experiment had a b-HMF concentration of 0.94 µg/l. By the end of incubation, this concentration had increased to 5.88 µg/l. The samples incubated in the presence of Faox I and II showed a decrease in the b-HMF concentration of 90 and 94 %, respectively.
Figure 3.5: Bound-HMF content in a UHT low lactose commercial milk at time zero (LLM T0) and after storage at 37 C for 17 days (LLM). Faox I or II were added immediately before storage in a 1:1,000 ratio. Mean change in bound HMF reduction was significant in relationship to the control after incubation using Duncan’s test (*p<0.05); n = 4.

When comparing the effect of Faox on the two different markers of the MR development it could be hypothesized that the more pronounced effect found on b-HMF as compared to that observed on CML might be due to the different formation pathways of these two compounds. In fact, CML could be formed reacting with lysine from both carbohydrates and lipids during autooxidation reactions, whereas b-HMF can only be derived from the Maillard reaction path.

The peptides of b-LG represent a marker to monitor the Faox activity

To verify the specificity of the enzyme action the samples of the β-LG-glucose model system at the end of the incubation time were subjected to cyanogen bromide hydrolysis and MS investigations. As showed in Figure 3.6, the hydrolysis of β-LG can lead to the formation of 5 peptides that were all found in the spectra (data not shown). Among them the peptides 1–7, 8–24 and the 108–145, which is present in two isoforms due to the two genetic variants β-LG-A and β-LG-B, were the most affected by Faox action. It is worth to be noticed that these target peptides, 8–24 and 108–
Fa ox enzymes inhibit development of the Maillard Reaction in storage both in the protein glucose model system and low lactose UHT milk.

145, contain lysine residues having high solvent accessibility, as reported previously.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Expected molecular mass (Da)</th>
<th>Aminoacid sequence</th>
<th>Control</th>
<th>Heat treated</th>
<th>FAOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-7)</td>
<td>756</td>
<td>LIVTQGIM</td>
<td>757</td>
<td>757</td>
<td>757</td>
</tr>
<tr>
<td>(8-24)</td>
<td>1833</td>
<td>KQLDIQVKAC TWYSLAM</td>
<td>1849</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>(25-107)</td>
<td>Var B</td>
<td>9332</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>(25-107)</td>
<td>Var A</td>
<td>9389.9</td>
<td>3464(Glu)</td>
<td>3464(Glu)</td>
<td></td>
</tr>
<tr>
<td>(108-145)</td>
<td>B (A1a)</td>
<td>4163.0</td>
<td>4286</td>
<td>4286</td>
<td>4288</td>
</tr>
<tr>
<td>(108-145)</td>
<td>B (A1a)1Glu</td>
<td>ENSAEPEGSGL ACQCLVRTPVEVDDEALEKFD KALKAPFM</td>
<td>4443</td>
<td>4450</td>
<td></td>
</tr>
<tr>
<td>(108-145)</td>
<td>B (A1a)2Glu</td>
<td>4612</td>
<td>4612.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(108-145)</td>
<td>A (Y1a)</td>
<td>4198</td>
<td>4315</td>
<td>4315</td>
<td>4317</td>
</tr>
<tr>
<td>(108-145)</td>
<td>B (A1a)1Glu</td>
<td>ENSAEPEGSGL VCGCLVRTPVEVDDEALEKFD KALKAPFM</td>
<td>4477</td>
<td>4479</td>
<td></td>
</tr>
<tr>
<td>(108-145)</td>
<td>B (A1a)2Glu</td>
<td>4637</td>
<td>4640</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(146-162)</td>
<td></td>
<td>2064</td>
<td>2122</td>
<td>2125</td>
<td>2125</td>
</tr>
</tbody>
</table>

Figure 3.6: Upper panel, amino acid sequence of bovine b-LG. Black arrows indicate CNBr hydrolysis sites. In yellow the amino acid substitution in variant A and B, putative glycated lysine residues are underlined. Bottom panel, expected and measured molecular mass after CNBr hydrolysis.

In Figure 3.7, the mass spectra of the region 730–2100 uma is showed highlighting the two smallest glycosylated peptides formed upon the cyanogen bromide hydrolysis namely the β-LG (f1–7) and β-LG (f8–24).
Figure 3.7: MALDI spectra of the region 730–2100 uma after CNBr hydrolysis β-LG after reduction and alkylation. Panels on the left showed the region of the fragment 1–7, panels on the right the region of the fragment 8–24. Top panel control samples without glucose; middle panel β-LG-glucose; bottom panel β-LG-glucose added with Faox I. Quantitative data are summarized in Table 3.1

Data of Table 3.1 showed that the amount of glycosylated form is reduced by 20 and 30 %. Similar results were also observed on the peptide β-LG (f108–145). This peptide is present in two isoforms related to the two genetic variants of β-LG (βLG-A and β-LG-B), therefore the use of smaller and unique peptide to monitor the Faox action was preferred. Interestingly, the reduction in glycosylation reaction caused by Faox measured on the glycosylated peptides of this model system is in line with that measured considering CML and b-HMF.
Table 3.1: Relative intensity values of CNBr peptides β-LG (1–7) and β-LG (8–24) from the MALDI-TOF analysis showed in Figure 3.7. RI (Relative intensity).

<table>
<thead>
<tr>
<th>β-LG Peptides</th>
<th>Molecular mass (Da)</th>
<th>Panel b (Control) RI</th>
<th>Panel c (Faox) RI</th>
<th>Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-LG (f1-7)</td>
<td>757.3</td>
<td>18.9</td>
<td>18.9</td>
<td>\</td>
</tr>
<tr>
<td>β-LG (f1-7) + Glu</td>
<td>919.3</td>
<td>8.5</td>
<td>6.8</td>
<td>20</td>
</tr>
<tr>
<td>β-LG (f8-24)</td>
<td>1832.7</td>
<td>100</td>
<td>100</td>
<td>\</td>
</tr>
<tr>
<td>β-LG (f8-24) + Glu</td>
<td>1995.7</td>
<td>5.2</td>
<td>3.5</td>
<td>30</td>
</tr>
</tbody>
</table>

Conclusions

In previous work by our group, the ability of Faox enzymes to inhibit MR development has already been shown.\textsuperscript{59, 170} Data demonstrated that when the enzyme is added together with glucose (i.e. during the glycation reaction) a reduction of protein glycation is observed by LC–ESI–MS/MS. This effect, although no clear mechanisms have been demonstrated, could be due to a temporary local unfolding of the protein which allows the glycated site to fit into the catalytic cavity of the enzyme. As expected in fact, when enzyme is added on already glycated b-LG, no effect is observed.\textsuperscript{170} This hypothesis is supported by the observation that using the enzyme at lower concentrations such as 1:2,000 and 1:5,000 the de-glycation action is severely reduced. This finding strengthened the hypothesis that Faox should react with the glycated protein before its refolding making the glycated site not accessible to the enzyme catalytic cavity. This paper is the first one comparing the effects of Faox I and Faox II both on a protein-glucose model systems and a real food substrate, such as the low lactose milk, considering MR markers, such as CML and b-HMF. Data presented here clearly showed that these enzymes can be used for limiting protein glycation that occurs during milk storage opening the way for a possible practical use during milk storage.
Chapter 4

Effect of olive mill wastewaters phenol compounds on reactive carbonyl species and Maillard Reaction end-products in ultra-high temperature treated milk

Antonio Dario Troise, Alberto Fiore, Antonio Colantuono, Smaro Kokkinidou, Devin G. Peterson, and Vincenzo Fogliano

Chapter 4

Effect of olive mill wastewaters phenol compounds on reactive carbonyl species and Maillard Reaction end-products in ultra-high temperature treated milk

Antonio Dario Troise, Alberto Fiore, Antonio Colantuono, Smaro Kokkinidou, Devin G. Peterson, and Vincenzo Fogliano

Thermal processing and Maillard reaction (MR) affect the nutritional and sensorial quality of milk. In this paper an olive mill wastewater phenolic powder (OMW) was tested as functional ingredient for inhibiting the MR development in UHT milk. OMW was added to milk at 0.1% and 0.05% w/v before UHT treatment and the concentration of MR products was monitored to verify the effect of OMW phenols in controlling the MR. Results revealed that OMW is able to trap the reactive carbonyl species such as hydroxycarbonyls and dicarbonyls, which in turn led to the increase of Maillard derived off-flavor development. The effect of OMW on the formation of Amadori products and Nε-(Carboxymethyl)-L-lysine (CML) showed that oxidative cleavage, C2-C6 cyclization and the consequent reactive carbonyl species formation were also inhibited by OMW. Data indicated that OMW is a functional ingredient able to control the MR and to improve nutritional and sensorial attributes of milk.

**Keywords:** Functional milk, olive oil phenols, Maillard reaction, reactive carbonyls species, olive mill wastewaters
Introduction

Thermal processing of foods guarantees milk safety and allows its long shelf life however it should be finely tuned to reach the equilibrium between the promotion of beneficial aspects and the reduction of thermal damages.\textsuperscript{186} The positive outcomes of food processing include the inactivation of food-borne pathogens or their toxins, the improvement of bioavailability and digestibility and shelf-life. Thermal processing may results in loss of texture and color, degradation of certain essential nutrients, formation of undesired compounds with negative sensorial properties and a potentially toxic effect on human health.\textsuperscript{28}

Many of the chemical modifications occurring during food thermal processing are connected to the Maillard reaction (MR). Maillard reaction products (MRPs) are formed by the reaction between reducing sugars and amino group counterparts;\textsuperscript{187} the formation of a glycosylamine is followed by the formation of two more stable intermediates: 1-amino-1-deoxy-2-ketose the Amadori rearrangement product (AP) and 2-amino-2-deoxyaldose the Heyns rearrangement product (HP).\textsuperscript{3} After this key step of the early stage of the MR a plethora of reaction can occur: sugar fragmentation and cyclization, Strecker’s aldehyde formation and degradation, retro-aldol condensation via Namiki pathway, pyrolysis, oxidative cleavage and polymerization. After the pivotal work by John Hodges,\textsuperscript{1} 70 years of research have well defined the main pathways of the MR and identified several molecules that can act as a markers of MR development such as lysylpyrraline, CML, CEL, pentosidine, furosine, hydroxymethylfurfural (HMF) and isomaltol.\textsuperscript{5, 163}

In milk products MR is not desired because of both sensory and nutritional reasons.\textsuperscript{13} Lactose derived Amadori compounds are the main MR products in milk products and their concentration increased according to thermal treatment and water activity (pasteurization > UHT > sterilization > milk powders) with a consequent blockage of the lysine residues and decrease of the nutritive value.\textsuperscript{188-190} Upon severe thermal conditions the breakdown of the Amadori products led to the formation of flavor compounds,\textsuperscript{191, 192} antioxidant and pro-oxidant compounds,\textsuperscript{193-195} polymerization of proteins and brown color development due to melanoidins generation.\textsuperscript{196-198}

Recently, the issue of safety and the health consequences related to the intake of these dietary advanced glycation end products (dAGE) has been raised.\textsuperscript{199, 200} Some papers highlighted the \textit{in vivo} diabetogenic and nephrotoxic effects of them and the term “glycotoxins” was even coined.\textsuperscript{201, 202 203} Although no firm conclusions have been achieved about the dAGE physiological
significance, the studies to explore the correlation between biomarkers related to the thermal impact of milk process and nutritional physiological and toxicological outcomes, are of great importance.\textsuperscript{163, 204, 205}

A characterization of food thermal damage can be achieved through the indirect measurement of the lysine APs such as lactulosyllysine or maltulosyllysine during acid hydrolysis by measuring furosine\textsuperscript{33, 206} or bound HMF.\textsuperscript{177, 207} More recently, some techniques allowed the direct measurement of the APs through LC/ESI-MS with or without labeled internal standard.\textsuperscript{3, 208-210} Other two routinely indicators of the thermal damage are CML and CEL that can be analyzed by MALDI-TOF/MS.\textsuperscript{211} LC-MS/MS after acidic hydrolysis stable isotope dilution assay \textsuperscript{178, 212} and GC/MS.\textsuperscript{213} This last technique is commonly used for the determination and the accurate quantification of MR volatiles compounds such as hydroxycarbonyls: glycolaldehyde, acetol and acetoine or off-flavor compounds such as methional and 2-acetyl-2-thiazoline.\textsuperscript{214-217} Last but not least, a broad range of publications focuses on the determination of \textsuperscript{13}C4-dicarbonyls, by using different derivatizing agents.\textsuperscript{218, 219}

The control of the MR in food using functional ingredients can promote the formation of compounds having the desired color and flavor and it can reduce the concentration of off-flavors and potential toxic molecules. During the last years several approaches have been proposed to inhibited MR development on milk,\textsuperscript{220} specifically by using green tea extract or catechins.\textsuperscript{221, 222} The mechanism behind the trapping activity of phenolic rings has been elucidated and the chemical structure of the epicatechin – methylglyoxal adduct has been identified.\textsuperscript{19, 223} Recently, response surface methodology has been used to investigate the dose response relationship of catechin, genistein and daidzein mixture as a pre-thermal processing technique to reduce the formation of reactive carbonyl species (RCSs) such as glyoxal, methylglyoxal and 3-deoxyglucosone in ultra-high temperature (UHT) milk.\textsuperscript{224} Twenty compounds having an aromatic ring substituted with at least one hydroxyl group have been reacted with methylglyoxal: results about the trapping efficiency can be transferred to milk product in order to verify the possibility to produce new better quality milk beverages.\textsuperscript{225}

Olive mill waste-water (OMW) is a by-product of olive oil production process and it represent an abundant sources of phenolic compounds.\textsuperscript{226} OMW typically contains 98% of total phenols originally present in the olive being only a minor part recovered in olive oil.\textsuperscript{227} OMW contained by more than 60 phenolic compounds among which the most relevant are secoiridoids derivatives,
such as hydroxytyrosol and the dialdehydic form of decarboxymethyl oleuropein aglycone along with tyrosol and verbascoside.\textsuperscript{228} The biological properties of its main components have been accurately reviewed.\textsuperscript{227} OMW has been proposed for a wide range of applications including the production of nutraceuticals,\textsuperscript{229} the formulation of fish feed,\textsuperscript{230} the stabilization during lard production,\textsuperscript{231} however its activity in preventing MR development in foods was never investigated.

In this paper, the ability of an ingredient obtained from OMW through ultrafiltration and successive spray-drying in controlling the MR in UHT milk was examined. OMW activity was tested in lab-scale UHT milk in order to verify its effectiveness in the reduction of early stage MR products, such as free APs, protein bounds APs and RCSs, in the reduction of off flavor formation, and in the inhibition of CML formation.

**Material and methods**

**Chemicals**

Acetonitrile, methanol and water for solid phase extraction (SPE) and LC-MS/MS determination were obtained from Merck (Darmstadt, Germany). The ion pairing agent perfluoropentanoic acid, trichloroacetic acid, hydrochloric acid (37%), sodium borohydrde, sodium hydroxide, the analytical standards L-lysine hydrochloride, [4,4,5,5-\textsuperscript{d}_4]-L-lysine hydrochloride (\textit{d}_4-Lys), tyrosol (2-(4-Hydroxyphenyl)ethanol, 98%), 3-hydroxytyrosol 98% and verbascoside 98% along with glyoxal 40% solution in water, methylglyoxal 40% solution in water, diacetyl (2,3-butanedione, 97%) as well as glycolaldehyde, acetoin, acetol and o-phenylenediamine (o-PD, 99.5%) and formic acid (MS grade 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Analytical standards Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML) and its respective deuterated standard Nε-(Carboxyl\textsuperscript{2H}_2)methyl)-L-Lysine (\textit{d}_2-CML) were obtained from Polypeptide laboratories (Strasbourg, France), while 3-deoxyglucosone and \textit{13}C\textsubscript{4}-acetoin and \textit{13}C\textsubscript{4}-diacetyl were obtained from TRC (Toronto research chemicals, Ontario Canada).

**Formulation of OMW**

Olive oil wastewater polyphenols powders (OMW) were prepared from the water fraction resulting from olive oil production as follows. Olive water were collected after olive paste centrifugation treated with pectinases and fractionated by a filtration plant made up with three
membranes at different cut off at 37 °C. Olive water was forced to pass through microfiltration (cut off 25 kDa), ultrafiltration (cut off 8 kDa), and nanofiltration (cut off 0.3 kDa) membranes. At each stage a retentate containing the compounds not passing the membrane pores was collected. The ultrafiltration (UFR) retentate was concentrated by inverse osmosis (cut of 0.1 kDa) up to 20% dry weight and spray dried adding maltodextrin and acacia fiber in a ratio 1:1 with the dry weight. A fine pale yellow powder with mild olive flavor was obtained and used in this study.

**OMW characterization**

The phenolic profile of OMW was characterized by reversed phase HPLC/UV-vis in order to quantify the phenylethanoids: hydroxytyrosol, tyrosol and verbascoside. Briefly, 100 mg of powder were dissolved in 10 mL of water and after the complete melting of the coating material (5 min, room temperature under continuous stirring), 1 mL was purified on a pre-activated C-18 cartridges (1 cc, 30 mg Phenomenex, Torrance, CA). Samples were eluted according to the method described by Kokkinidou and Peterson224 and after drying they were re-constituted in a mixture of water/methanol 95:5 (v/v) and 20 µL were injected onto a Prodigy ODS3 250 mm x 4.6 reversed phase C-18 (Phenomenex, Torrance, CA). The UPLC system consisted in two binary pumps (LC-20A, Shimadzu, Kyoto, Japan) equipped with an UV-Vis detector (SPD20A, Shimadzu, Kyoto, Japan) The mobile phases were water 0.1% formic acid (A) and methanol (B). Hydroxytyrosol, tyrosol and verbascoside were separated according to the following gradient ([min]/[%B]): (0/5), (5/5), (40/70), (42/70), (45/5), (50/5) and the typical retention times were 20, 23 and 28 min, respectively. The three analytes were quantified by the external standard technique and the results reported as mg/g of powder.

**Lab scale UHT Milk treatment**

Raw milk (protein 3.5%, fat 1%) was obtained from a local market and OMW were added in order to obtain a final concentration of 0.5 mg/mL and 1 mg/mL. The three samples, one control and two samples with OMW were homogenized and after the complete dispersion of the powder (5 min, room temperature under continuous stirring) thermally treated in a lab scale UHT milk system. It was constituted by three different tanks: two with oil, set at 180 °C and 140 °C respectively, and another one with water at 6 °C. All the samples were simultaneously treated and one vial was used as reference. Along with 5 mL of milk a thermocouple was inserted inside the reference head-space vial in order to control the time/temperature profile of each set. The high
temperature of the first batch allowed a rapid increase of the temperature in the vial, instead the second tank stabilized the temperature at 140 °C for 5 s. At the end of the thermal process the samples were rapidly moved into the last tank where the cooling phase blocked the extent of the MR. Finally, as soon as the samples reached 15 °C, they were rapidly frozen in dry ice in ethanol and stored at -20 °C until the analysis.

**CML, total lysine and furosine analysis**

Total lysine and its derivatives Nε-(Carboxymethyl)-L-lysine (CML) and Nε-(2-Furoylmethyl)-L-lysine (furosine) were analyzed according to Fenaille et al.; Delatour et al.; and Troise et al.; with some modifications. Briefly, 100 µL of milk were mixed along with 0.45 mL of sodium borohydride (1 M in 0.1 N NaOH) and 0.5 mL of borate buffer (pH 9.2). The mixture was incubated for 4 h at room temperature in order to reduce the fructosyl-lysine in hexitol-lysine, and 1 mL of TCA (20% final concentration) was added in order to promote protein precipitation. Finally 2 mL of 6 N HCl were added after careful removal of the supernatant. The mixture was incubated for 24 h at 110 °C in an air forced circulating oven and 400 µL were evaporated under a gentle flow of nitrogen. The samples were reconstituted in 380 µL of water and 10 µL of the internal standard d2-CML and 10 µL of d4-Lysine were added in order to obtain a final concentration of 50 ng/mL for both standards. Samples were loaded onto equilibrated Oasis HLB 1 cc cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described, finally 5 µL were analyzed by ion pairing liquid chromatography coupled to MS/MS. Furosine, CML, lysine and the respective internal standards separation was achieved on a reversed–phase core shell column (Kinetex C18, 2.1 mm x 100 mm, Phenomenex, Torrance, CA) with a C-18 pre-column (3.0 x 4.0 mm Phenomenex, Torrance, CA) using the following mobile phases: A, 5 mM perfluoropentanoic acid and B, acetonitrile 5 mM perfluoropentanoic acid. Compounds were eluted at 200 µL/min through the following gradient of solvent B [(min)/[%B]]: (0/10), (2/10), (5/70), (7/70), (9/90), (10/90), (12/10), (15/10). With the above described chromatographic conditions, typical retention time of CML and d2-CML was 6.8 min, for d4-Lysine and lysine was 7.03 min and for furosine was 7.31 min. The MS/MS system was an API3000 (Applied Biosystems, Carlsbad, CA) and positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage: 5.0 kV; capillary temperature: 350 °C, dwell time 100 ms. The chromatographic profile was recorded in multiple reaction monitoring mode and the characteristic transitions were monitored in order to improve selectivity: for CML, furosine and lysine, the
respective transitions of $m/z$ 205-84.1, $m/z$ 255.1-130.2 and $m/z$ 147.2-130.2 were used as quantifier whereas $m/z$ 205-130.2, $m/z$ 255.1-84 and $m/z$ 147.2-84.1 were used as qualifier. CML was quantified using $d_2$-CML as internal standard ($m/z$ 207-144.1 and $m/z$ 207-84 for quantification and confirmation, respectively), while for furosine and lysine, $d_4$-Lysine was used ($m/z$ 151.2-134.1 and $m/z$ 151.1-88 for quantification and confirmation, respectively).

**Free Amadori products**

For the free Amadori products (APs) detection each milk sample was diluted ten times with water and ultracentrifuged (14800 rpm 10 min 4 °C), then the supernatants were accurately filtered (RC 0.45 µm, Phenomenex, Torrance CA) and injected. For the chromatographic separation a reversed–phase core shell column (Kinetex C18, 2.1 mm x 100 mm, Phenomenex, Torrance, CA) with a C-18 pre-column (3.0 x 4.0 mm Phenomenex, Torrance, CA) was used. The mobile phases consisted in 5 mM NFPA in water (solvent A) and 5 mM NFPA in acetonitrile (solvent B). The following gradient of solvent B (t in [min]/[%B]): (0/10), (2/10), (5/50), (7/50), (9/10), (12/10), (15/10) was used. The flow rate was set to 200 µL/min and the injection volume was 5 µL. The U-HPLC was directly interfaced to an Exactive Orbitrap high resolution mass spectrometer (HRMS) equipped with a heated electrospray interface (HESI). Mass spectrometer operated in the full spectra positive ionization acquisition mode, in the mass range of $m/z$ 65–500; the mass spectrometry parameters were set up according to Troise et al.\(^{232}\)

**Quantification of reactive carbonyl species (RCSs)**

Methylglyoxal, glyoxal, 3-deoxyglucosone and diacetyl (2,3-butanedione) as well as glycolaldehyde, acetoin and acetol were quantified using the synthesized stable isotopes $^{13}$C$_4$-acetoine and $^{13}$C$_4$-diacetyl as internal standards. Each internal standard (0.5 mM) was added to 5 mL of milk, followed by 500 µL of 10% trichloroacetic acid. The samples were then vortexed and centrifuged at 3904 g for 20 min at 4 °C (Beckman Coulter, Allegra X-22R) and the supernatant was collected. Solid Phase Extraction (SPE) method was used to isolate the compounds of interest and avoid interference from phenolic compounds and adducts. A derivatization method followed and samples were analyzed using an Acquity UPLC system interfaced with a Quattro Premier XE micromass mass spectrometer (Waters Co. Milford, MA). An Acquity UPLC 2.1x100mm BEH Phenyl 1.7 µm column with a VanGuard 2.1 x 5 mm BEH Phenyl 1.7 µm pre-column were used for separation and all experiments were performed in triplicate. Analytes were detected using
electrospray positive ionization–multiple reaction monitoring (MRM) using methods previously developed and reported by Kokkinidou and Peterson and is specifically design to avoid quantitative interference from phenolic compounds and adducts.\textsuperscript{224}

**Quantification of Maillard related off-flavor volatiles**

A dynamic headspace method was developed for the quantification of off-flavor markers, namely 2-acetyl-2-thiazoline and methional. Analysis was performed using a 6890 GC equipped with a 5973 Mass Selective Detector (Agilent Technologies), Thermal Desorption Unit (TDU, Gerstel), PTV inlet (CIS 4, Gerstel) and MPS 2 (Gerstel). Briefly, 2 mL of nanopure water and 1 M of sodium chloride were added to 5 ml of UHT milk (control and treatments) spiked with deuterated standards. Sample was then purged with nitrogen, dry-purged to eliminate water and was then transferred to a thermal desorption unit. Measurements were performed 24 h after UHT processing using the analytical parameters summarized in Table 4.1.

**Statistical analysis**

Each thermal treatment was repeated twice in order to improve the reliability of the lab scale UHT system, while each sample was analyzed twice and inject two times. Results were reported as ng/mL of milk for RCSs, mg/100 g protein for furosine, lysine and CML and % inhibition towards the control samples for free APs. Data were analyzed by ANOVA, and means were compared by Tukey test ($\alpha = 0.05$) using XLStat statistical software (Addinsoft, New York, NY).
Table 4.1: Analysis conditions used for identification and quantification of Maillard-related off-flavor markers.

<table>
<thead>
<tr>
<th>Analysis conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTV</strong></td>
</tr>
<tr>
<td>Solvent vent (20 mL/min) at 7.1 psi</td>
</tr>
<tr>
<td>Solvent vent (0.5 min), 20 °C (0.5 min); 10 °C/s; 290 °C (4 min)</td>
</tr>
<tr>
<td><strong>Column</strong></td>
</tr>
<tr>
<td>DBS-MS 30m x 0.25 mm x 0.25 μm; He, constant flow = 1 mL/min</td>
</tr>
<tr>
<td><strong>Oven</strong></td>
</tr>
<tr>
<td>40 °C (2 min); 4 °C/min; 160 °C; ;30 °C /min; 250 °C (5 min)</td>
</tr>
<tr>
<td><strong>MSD</strong></td>
</tr>
<tr>
<td>SIM, 104+48 (methional), 107+51 (d3-methional), 129 (2-acetyl-2thiazoline), 133 (d4-2-acetyl-2thiazoline)</td>
</tr>
<tr>
<td>Dynamic headspace component</td>
</tr>
<tr>
<td><strong>Chemical trap</strong></td>
</tr>
<tr>
<td>Tenax TA</td>
</tr>
<tr>
<td><strong>DHS unit</strong></td>
</tr>
<tr>
<td>Trap temperature: ambient , 40 °C sample incubation temperature (10 min)</td>
</tr>
<tr>
<td>1000 mL purge volume, 50 mL/min purge flow</td>
</tr>
<tr>
<td>320 mL dry volume, 40 mL/min dry flow</td>
</tr>
<tr>
<td><strong>TDU unit</strong></td>
</tr>
<tr>
<td>Solvent venting 20 °C (1 min); 720 °C/min; 290 °C (4 min)</td>
</tr>
</tbody>
</table>

Results and discussion

**OMW characterization and thermal treatment**

The use of a OMW ingredient obtained from ultrafiltered and spray-dried olive wastewater was evaluated for its ability to control the overall extent of the MR. The functional aspects of OMW were tested using a lab-scale UHT milk and the selected outcomes were the MR aroma key odorants, the reduction of early stage MRPs such as APs; the protein bound MRPs formation such as furosine and CML and the ability to trap RCSs.

The first part of the work involved the production of OMW by four subsequent steps: the starting material was made by the water resulting after oil separation which was treated with pectinas and fractionated by three different membranes to obtain three different retentate fractions.\(^{233,234}\)

The ultrafiltration retentate was concentrated by inverse osmosis up to 67% dry matter and spray dried with acacia fiber and maltodextrin in molar ratio 1:1 in order to obtain a fine pale yellow powder.\(^{85}\) The phenolic profile of OMW was characterized by reversed phase UPLC/UV-vis in order to quantify the main constituents: hydroxytyrosol, tyrosol and verbascoside. The concentration of the three compounds in the OMW were: 31 ± 0.2 mg/g ; 1.9 ± 0.1 mg/g; and 2.8 ± 0.09 mg/g, respectively in line with previous paper dealing with composition of OMW.\(^{226,228,235}\)
Two different concentrations of OMW (0.05 % and 0.1 % w/v) were dissolved in raw cow milk and their impact on the thermal damage was investigated by monitoring several intermediates and end products of MR. A lab-scale UHT treatment was developed for this purpose: the thermal profile of the treatment confirmed that the thermal load closely simulated those of a commercial indirect tubular UHT processing method as highlighted in Figure 1.

![Time/temperature profile](image)

**Figure 4.1:** Comparison between lab scale UHT system and commercial indirect tubular UHT system. The holding time used was the same for direct UHT processing and UHT plaque sterilization.

In the system here developed the time/temperature profile was similar to the ones previously reported for commercial indirect tubular UHT processing, direct UHT processing and plaque UHT sterilization.\textsuperscript{236, 237} The sterilization factors were calculated according to Morales et al. using as reference temperature 127 °C and as z value 30 °C.\textsuperscript{183} They were 1.55 min and 1.51 min for commercial UHT milk and lab scale UHT milk, respectively. In order to compare the two concentrations of OMW a control sample (without OMW) was simultaneously heated and several MRPs and RCSs were measured. In this conditions the final concentration of olive polyphenols reached a maximum of 36.1 mg/L without evident effects on milk sensory properties.

**Off-flavor in UHT milk**

Off-flavor formation in UHT milk was already investigated in different papers.\textsuperscript{191, 238, 239} According to the previous literature methional and 2-acetyl-2-thiazoline, commonly used as marker of off-flavor formation or aroma active compounds, were selected to verify the activity of OMW on this parameter. As shown in Figure 4.2 the concentration of methional was reduced from 4.08 ± 0.33
ng/mL for control UHT milk to 3.09 ± 0.26 ng/mL and 2.15 ± 0.28 ng/mL, for OMW 0.05% and OMW 0.1%, respectively. Furthermore the concentration of 2-acetyl-2-thiazoline was reduced from 0.87±0.09 ng/mL for control UHT milk to 0.74 ± 0.11 ng/mL and 0.48 ± 0.08 ng/mL, for OMW 0.05% and OMW 0.1%, respectively. This means that compared to control UHT milk OMW 0.05% reduce by 24.3 and 16.9%, the concentration of methional and 2-acetyl-2-thiazoline, respectively, albeit that reduction was not statistically significant. Increasing the OMW concentration to 0.1% the concentrations of methional and 2-acetyl-2-thiazoline were found to reduce by 47.3 and 46.1% respectively when compared to control UHT milk.

![Figure 4.2](image)

**Figure 4.2:** Methional and 2-acetyl-2-thiazoline concentration in control UHT milk, milk with 0.05% OMW and milk with 0.1% OMW. Different letters correspond to significant differences (Tukey test, α= 0.05).

This reduction was significant indicating a concentration-dependent effectiveness of the phenolic mixture in reducing off-flavor generation during thermal processing. Data clearly showed that increasing the added OMW concentration prior to thermal processing can significantly decrease the concentration of off-flavor markers when compared to control UHT milk. These results were in line with those previously reported about the milk off-flavor and the potential role of added polyphenol.239-242

**Trapping activity towards RCSs**

The trapping activity of OMW towards RCSs was evaluated by monitoring the concentration of hydroxycarbonyl and dicarbonyl compounds in UHT milk with and without added OMW prior to thermal processing. The target RCSs monitored were glycoaldehyde, acetoaldehyde, acetoin and acetol for hydroxycarbonyls and glyoxal, methylglyoxal, 3-deoxyglucosone and 2,3-butanedione for α-dicarbonyls as they have been extensively studied for their contribution to AGEs generation and
carbonyl stress. Results are presented in **Figure 4.3** and **Figure 4.4**: the concentration of glycoaldehyde decreased from 246.03 ± 26.74 ng/mL to 135.71 ± 30.63 ng/mL and 105.36 ± 25.11 ng/mL for control UHT milk, OMW 0.05% and 0.1%, respectively. Corresponding to a reduction of 44.8% and 57.2% for samples containing OMW 0.05% and 0.1%, respectively. Similarly, OMW reduced the concentration of acetoin from 2293.73 ± 165.35 ng/mL to 1897.91 ± 201.41 ng/mL and 1321.86 ± 86.11 ng/mL for control UHT milk, OMW 0.05% and 0.1%, respectively. Corresponding to a reduction of 17.3 and 42.4% of acetoin for OMW 0.05% and 0.1% treatments. On the contrary, OMW did not show any reactivity towards acetol as no significant difference was observed between control UHT milk, and OMW samples. These results can be explained on the basis of chemical reactivity as it follows the electrophilicity of hydroxycarbonyl compounds and thus the reactivity of phenolic compounds towards trapping them: the electron rich phenols present in OMW can easily react with electrophilic hydroxycarbonyl compounds due to the presence of hydroxyl group in position α.

Slight differences in the concentration of α-hydroxycarbonyls towards previously reported papers can be ascribed to external factors such as microbial activity, starting material, thermal treatments. ²⁴¹-²⁴³

**Figure 4.3:** Hydroxycarboxyls concentration in control UHT milk, milk with 0.05% OMW and milk with 0.1% OMW. Different letters correspond to significant differences (Tukey test, α=0.05). GA = glycoaldehyde.
The concentration of glyoxal was reduced from 2118.71 ± 113.94 ng/mL in control UHT milk to 1798.04 ± 218.49 ng/mL and 1201.36 ± 136.73 ng/mL adding OMW at 0.05% and 0.1%, respectively. This means a reduction of 15.1% and 43.3% for glyoxal in samples added with OMW 0.05% and 0.1%, respectively. Methylglyoxal values were also reduced of 23.8% and 55.9% by OMW 0.05% and 0.1% addition, respectively. Both electrophilic dicarbonyls can give hydroxyalkylation of phenolic rings: Figure 4.4 suggested a higher activity of OMW towards methylglyoxal respect to glyoxal and this can be also due to the fact that the latter can polymerize in aqueous media resulting in a lower affinity towards phenols.\textsuperscript{244} For both compounds the observed reductions were in line with those previously reported.\textsuperscript{224,225}

The concentration of 2,3-butenedione was reduced from 11.79 ± 0.55 ng/mL for control UHT milk to 9.16 ± 1.04 ng/mL and 5.98 ± 0.09 ng/mL for OMW 0.05% and 0.1%, respectively and 3-deoxyglucosone was modified only by addition of higher concentration of OMW as no significant difference was observed between control UHT milk and milk treated with OMW 0.05%. It can be hypothesized that the trapping activity toward 3-deoxyglucosone is affected by the partial charge on the oxygen of the carbon carbonyl in position 2, by the presence of other carbons that can stabilize the structure and by the steric hindrance. From this first set of experiments it can be concluded that OMW treatment was not very effective in trapping C6 sugar fragments while a significative reactivity of phenolic compounds in OMW mixture was observed towards methylglyoxal, diacetyl and glycoaldehyde.

All the RCSs monitored are of interest not only for their contribution to MR pathways and carbonyl stress, but also because they can contribute to the generation of off flavor compounds thus, their suppression can both directly and indirectly led to a reduction in off-flavor generation.
The concentration of 2,3-butenedione was reduced from 11.79 ± 0.55 ng/mL for control UHT milk to 5.98 ± 0.09 ng/mL for OMW 0.05% and 3- to 9.16 ± 1.04 ng/mL and 5.98 ± 0.09 ng/mL for OMW 0.05% and 0.1%, respectively. The concentration of glyoxal was reduced from 2118.71 ± 113.94 ng/mL in control UHT milk to 1179.04 ± 218.49 ng/mL and 1201.36 ± 136.73 ng/mL adding OMW at 0.05% and 0.1%, respectively. This means a reduction of 15.1% and 43.3% for glyoxal in samples added with OMW 0.05% and 0.1%, respectively.

Methylglyoxal values were also reduced of 23.8% and 55.9% by compared to control UHT milk. This means a reduction of 15.1% and 43.3% for glyoxal in samples added with OMW 0.05% and 0.1%, respectively.

Figure 4.4: α-dicarbonyls concentration in control UHT milk, milk with 0.05% OMW and milk with 0.1% OMW. Different letters correspond to significant differences (Tukey test, α = 0.05).

Amadori compounds reduction

The effect of OMW was tested also in the reduction of free APs, which can be measured without protein hydrolysis, thanks to the relatively high content of free amino acids in milk. The formation of the AP was the central hub for the forthcoming steps of MR, so the presence of the phenolic rings favored the reduction of the APs formation and in turn of many MRPs via direct reaction with amino-group or via glyoxal trapping.

As shown in Figure 4.5 the reduction of APs formation was particularly evident for hydrophobic glycated amino acids, such as isoleucine/leucine, and glycine. This can be explained with the high concentration of these amino acids and their APs and with the lower reactivity than other amino acids towards reducing sugars. The best known compounds are the Amadori product Nε-fructosyllysine and furosine. The effect of OMW on free Nε-fructose-lysine formation was significant at both concentration: 12.1% and 17.8% over the control sample for OMW 0.1% and OMW 0.05%, respectively.

Figure 4.5: Effect of olive mill wastewaters phenol compounds on Amadori compounds reduction in ultra-high temperature treated milk.
Finally the ability of OMW to reduce the formation of proteins bound CML and furosine was investigated. Results in Figure 4.6 showed that after acidic hydrolysis the reduction of protein bound MRPs was more pronounced for furosine where the reduction was 25.4% and 47.4% when OMW 0.05% and OMW 0.1% of phenolic powder were added, respectively. For CML the reduction was 11.2% and 16.2% showing significant differences between the two phenolic powder concentrations and the control samples. Data on furosine showed that its concentration decreased from $10.962 \pm 0.805$ mg/100g protein in the control UHT milk, to $8.162 \pm 0.794$ mg/100g protein and $5.761 \pm 0.924$ mg/100g protein for OMW 0.05% and 0.1%, respectively.

**Control of bound MRPs formation**
The differences between CML and furosine reduction can be tentatively explained in two ways. Firstly, the two MRPs could follow two different pathways. Furosine is formed by cyclization through C-6 hydroxyl group and dehydration reaction and it reflects the proteins bound Amadori products of lysine, since the free Ne-fructose-lysine is destroyed by the acidic hydrolysis. CML can be formed by two mechanisms: the enediol form of the Ne-fructosyl-lysine can undergo oxidative cleavage to produce CML and erythronic acid via free radical generation with simultaneous oxygen consumption and glyoxal can easily block the amino group on the side chain via Namiki pathway. Secondly, as shown in Figure 4.7 the reduction of both proteins bound and free MRPs can be associated not only with the reduction of the Amadori product of lysine and glucose, but also to the above described trapping activity. Tri-substituted phenolic ring with ortho-dihydroxy function can easily react with dicarbonyls by hydroxyalkylation and aromatic substitution reaction thus showing trapping activity towards methylglyoxal and glyoxal.
Moreover, in the presence of reactants such as ascorbic acid or iron the phenolic ring can be oxidized to quinone, whose reaction with side chain of lysine and other amino group can lead to the formation of iminoquinone and iminophenol via Schiff bases as highlighted by Estévez and Guerra and Yaylayan. The results on CML and furosine fully confirmed those previously obtained by other group.

Conclusions

In conclusion, in this paper it was demonstrated that OMW beside its potential nutritional functionality could also help in the production of superior quality food and can provide an effective strategy in the control of the MR. The result was achieved on a broad range of MRPs which contributed to the final chemical composition of the UHT milk. Interestingly, these results were obtained in conditions do not leading to easily detectable modifications of both physical properties (viscosity or color) or sensory properties (bitterness, astringency, olive flavor). However the sensory acceptability of the addition of OMW should be further investigated.
Chapter 5

Amadori products formation in emulsified systems

Antonio Dario Troise, Claire C. Berton-Carabin and Vincenzo Fogliano

Submitted for publication
The control and the modulation of the Maillard Reaction (MR) in foods is a priority as its chemical pathways can lead to the formation of either potentially toxic molecules or desired flavored and colored compounds. The formation of Amadori Products (APs) is the key step determining the development of the MR: it has been widely studied in water or in dry systems, however available information on the chemical behavior of amino acids and reducing sugars in emulsified systems during thermal treatments is scanty and only focused on the volatiles compounds. Emulsified systems are useful not only as model food matrices, but also as micro-reactors to control chemical reactions, as their multiphase structure induces specific partitioning of the reactants. The aim of this work was to investigate the formation of APs from glucose and two amino acids with different partition coefficients in emulsion systems. A submicron oil-in-water (O/W) emulsion consisting of water, glyceryl trioctanoate (10% w/w) and Tween 20 (1% w/w) was prepared and the formation of the APs from phenylalanine and leucine (N-(1-deoxy-D-fructos-1-yl)-L-phenylalanine (Fru-Phe), and N-(1-deoxy-D-fructos-1-yl)-L-leucine (Fru-Leu), was monitored by high resolution mass spectrometry. The extent of Fru-Phe formation in microemulsion was similar to that in water, while that of Fru-Leu was reduced up to 47% in the microemulsion systems. These data indicated that partition coefficient of amino acids, determining the reactants location, can profoundly influence the final extent of the MR in emulsified systems. Investigations of the MR in emulsified systems can extend the possibility to improve the quality features of thermally treated foods and emulsions containing ingredients.

**Keywords:** Amadori products, Maillard reaction, microemulsion, partition coefficient, high resolution mass spectrometry.
Introduction

The Maillard reaction (MR) largely determines the final quality of foods by tuning the development of attributes such as color, flavor and nutritional values. The complexity of the MR chemical networks leads to the following dichotomy: on the one hand it promotes the formation of desired aroma and colors, but on the other hand it causes the formation of potentially toxic molecules which can affect the safety of the product. Thereby, the MR directly influences two quality pillars of food product: the ability of to satisfy consumer sensory expectations and the absence of compounds with potential negative effects on health.

The reaction between reducing sugars and amino groups results in the formation of the Amadori products, the central hub from which a myriad of reactions starts off. It has been established that the initial amino-carbonyl condensation forms a labile N-substituted D-glycosylamine, which undergoes the Amadori rearrangement to form the respective N-substituted D-fructosamine. Specifically, after the nucleophilic addition, the Schiff base can rearrange via 1,2-eneaminols which leads to an aminoketose, or Amadori rearrangement product (1-amino-1-deoxyketose, AP). If fructose reacts in a similar way with an amine or an amino acid, an aminoaldose (Heyns rearrangement compound, 2-amino-2-deoxyaldose, HP) can be formed. Since the pivotal Hodge’s paper about the APs role, a huge variety of papers have dealt with the significance of the molecules formed by its degradation. Up to now, the MR has been mainly studied in aqueous media and less frequently in dry systems, while the information available regarding the development of MR in complex colloidal systems, such as emulsions systems, were primarily focused to flavor compounds formation. In emulsion systems, environments with various properties and polarities are available, promoting the partitioning and segregation of the reactants (in the case of the MR, amino acids and sugars) and reaction products, according to their molecular structures and partition coefficients.

An emulsion consists of two immiscible liquids, with one of the liquids dispersed as small spherical droplets in the other. When oil is the dispersed phase, oil-in-water (O/W) emulsions are formed, which represent a variety of lipid-containing foods such as milk, dairy products, salad dressings, mayonnaise, sauces, soups, beverages, and cream. According to the droplet size and to the thermodynamic stability, emulsions can be classified into nanoemulsions and microemulsions. A nanoemulsion is a conventional thermodynamically unstable emulsion, where the mean radii of
the droplets is between 10 and 100 nm. Microemulsions are characterized by narrow particle size distributions, a structure that does not change during prolonged storage, and a mean radius in the range 2-50 nm.\textsuperscript{257, 258}

Food emulsions may not only be subjected to physical destabilization, but their major components (i.e., proteins, lipids and polysaccharides) and their inner structures with microscale and nanoscale compartments may also undergo chemical degradation because of thermal treatments, shelf-life and end use.\textsuperscript{259} Specifically, in emulsion environment the conventional relationship between reactants and products should also consider the reactants’ partition coefficients between the available phases. One of the most common examples is the study of the antioxidants polarity which determines their partitioning between oil, water and interfacial regions and their reactivity.\textsuperscript{260} Lipid oxidation is a well-studied case in this respect: the large surface area of dispersed droplets facilitates interactions between the lipids and water-soluble prooxidants and the total extent of the reaction is mediated by several properties related not only to the ingredients or target compounds but also to pH, particle size, concentration, physical state, oxygen and light.\textsuperscript{142, 261, 262} As lipid oxidation and MR have common intermediates (e.g. reactive carbonyls) and interrelated polymerization mechanisms in an emulsion containing MR reactants subjected to thermal treatment it is possible that both reactions should be considered simultaneously.\textsuperscript{263, 264}

A field of specific interest for investigating MR in the emulsions has been flavor development. Several authors have used structured self-assembled liquids as efficient microreactors for flavor development.\textsuperscript{83} They studied the development of Maillard volatile products in binary structured fluids composed of monoglycerides of fatty acids and water forming microemulsions and lyotropic liquid crystalline structures; pseudoternary and pseudoquaternary W/O microemulsions; U-type microemulsions (water-in-oil W/O, O/W and bicontinuous microemulsions). In such systems, the combination of reduced water activity and the presence of surfactants revealed a reaction rate slower than those carried out in aqueous solution and the formation of unique aroma compounds.\textsuperscript{83, 265, 266} The impact of emulsions combined with the thermal treatment has been evaluated also ghee production: authors found that the ratio and the type of volatile compounds produced in cooked samples can be altered by switching the dispersed phase from oil to water.\textsuperscript{267}

Tailored emulsified system can be proposed to control the MR development, not only for flavor, but also for non-volatile compounds responsible for color and for the generation of potentially harmful products. The possibility to control the MR by encapsulation was previously proposed
including sodium chloride into lipid capsules to delay its catalytic action.\textsuperscript{167, 268, 269} The research hypothesis is that also in the emulsion system it is possible to modify reactants location modulating the MR accordingly.\textsuperscript{220} In fact, the presence of oil could favor the location of some reactants at the oil-water interface, thus modulating the final extent of the Maillard reaction products (MRPs) formation, compared to a pure aqueous system.

In the present paper, an experimental design using two amino acids of different polarities and partition coefficients was developed to investigate the formation of N-(1-deoxy-D-fructos-1-yl)-L-phenylalanine (Fru-Phe) and N-(1-deoxy-D-fructos-1-yl)-L-leucine (Fru-Leu) in O/W sub-micron emulsion systems obtained by using high pressure homogenization. High resolution mass spectrometry data allowed to verify the influence of this system on the formation of APs during thermal treatment. Based on these findings, an overview of the reaction mechanisms leading to MRPs formation in emulsion systems is proposed.

**Material and methods**

**Chemicals and reagents**

Mass spectrometry grade acetonitrile and water were obtained from Merck (Darmstadt, Germany). The chemicals for the preparation of the model systems, L-leucine (Leu), L-phenylalanine (Phe), glyceryl trioctanoate (tricapryl), poly(oxy-1,2-ethanediyl)-sorbitan monododecanoate (Tween 20), benzoic acid, sulphuric acid, glucose oxidase/peroxidase reagents kit (GAGO-20) and o-dianisidine were all obtained from Sigma-Aldrich (St. Louis, MO). N-(1-deoxy-D-fructos-1-yl)-L-phenylalanine and N-(1-deoxy-D-fructos-1-yl)-L-leucine were obtained from Toronto Research Chemicals (Toronto, Canada). The MS calibration solution was obtained from Thermo Fisher (Bremen, Germany).

**Model systems preparation**

Two control aqueous solutions of D-glucose (13.8 mM) were separately mixed with Leu (4.5 mM, partition coefficient log\textsubscript{P}: 0.799 ± 0.275) and Phe (6.5 mM, partition coefficient log\textsubscript{P}: 0.235 ± 0.277). Both solutions (Leu/glucose and Phe/glucose) were stirred for 30 min at room temperature at 800 rpm. For the preparation of O/W microemulsions, an aliquot of the two control solutions was mixed with Tween 20 (final concentration in the emulsion system, 1% w/w). A coarse
emulsion was prepared by mixing 10% (w/w) oil phase and 90% (w/w) aqueous phase, then the solution was sonicated (1 min) by using a water bath, finally it was blended by a high-shear mixer at 22,000 rpm for 40 s, three times. Fine emulsions containing glyceryl trioctanoate were obtained by passing the coarse emulsion three times through a high pressure homogenizer (microfluidizer M110Y, Microfluidics, Newton, MA, configured with 75- and 200-µm interactions chambers in series) working at 30,000 psi. The same process was also applied for Leu and Phe control aqueous solutions to take into account any interference due to the process, as highlighted in Figure 5.1.

**Thermal treatment**

The four samples (two control aqueous solutions and their counterpart emulsions) were stored overnight at 4 °C, then they were thermally treated. Five milliliters of the two control samples and the two emulsified samples were introduced in a screw capped flask, then they were placed in a Liebisch 33 heater block (Bielefeld, Germany) for 2, 4, 6 and 8 min at 131 °C, a temperature close to the ultrahigh temperature treatment of milk, one of the most common processes used in the food industry. After each cycle the system was equilibrated at 131 °C for 5 min before treating the next sample. Before the thermal process (time 0) and at the end of each step, an aliquot of the samples was immediately frozen in an ice bath and stored at -40 °C until the high resolution mass spectrometry analysis, while 2 mL were directly used for the emulsion characterization.
Emulsion was prepared by mixing 10% (w/w) oil phase and 90% (w/w) aqueous phase, then the solution was sonicated (1 min) by using a water bath, finally it was blended by a high-shear mixer at 22,000 rpm for 40 s, three times. Fine emulsions containing glyceryl trioctanoate were obtained by passing the coarse emulsion three times through a high pressure homogenizer (microfluidizer M110Y, Microfluidics, Newton, MA, configured with 75- and 200-µm interactions chambers in series) working at 30,000 psi. The same process was also applied for Leu and Phe control aqueous solutions to take into account any interference due to the process, as highlighted in Figure 5.1.

**Figure 5.1:** Experimental set up of the emulsion preparation. The same process was applied to aqueous model system and emulsions. The lipidic phase in yellow consisted in glyceryl trioctanoate (10%, w/w) and Tween 20 (1% w/w). Reactants (glucose and amino acids) were dissolved in the water phase in blue. Mechanical treatment lead to the formation of an emulsion in gray. The thermal treatment was applied for 2, 4, 6 and 8 min at 131 °C. The emulsion remained stable also after the thermal treatment (see figure 5.2 and table 5.1)

**Emulsions characterization**

The mean particle diameter and particle size distribution of the emulsions were measured before and after the thermal treatment by using static light scattering (Mastersizer 2000, Malvern Instruments, Malvern, U.K.). The samples were diluted in distilled water at ambient temperature (20 °C) prior the analysis to avoid multiple scattering effects. The refractive indices used for the analysis were 1.45 for the dispersed phase (glyceryl trioctanoate) and 1.33 for the dispersant (water). The interfacial area per mass unit of dispersed phase, or specific surface area (A, m²/g oil) was calculated according to the equation 1.

\[ A = \frac{3}{r \times \rho} \]  

(1)
Microscope analysis

An optical microscope with a 40x objective lens (Carl Zeiss, Jena Germany) was used to capture images of the emulsion samples before and after the thermal treatment. A drop of emulsion was placed between a glass slide and cover slip, the slide was loaded onto the microscope stage, and then images were recorded in various locations of the sample.

Amadori compounds and amino acids analysis

The frozen O/W emulsion systems and the control aqueous samples were shaken at 1500 rpm for 10 min, then centrifuged at 14,800 rpm for 20 min at 0 °C in order to separate the creamed phase (if any) from the aqueous phase and to favor the passage of the polar target compounds in the aqueous layer. Samples were diluted 50 times in water before the analysis carried out by an Exactive Orbitrap high resolution mass spectrometer (HRMS, Thermo Fisher, Bremen, Germany). The concentration of APs was monitored according to Troise and co-workers with slight modifications. For the liquid chromatography separation of Leu, Phe, Fru-Leu and Fru-Phe, an Accela 1250 ultra-high pressure liquid chromatography system (UPLC, Thermo Fisher, Bremen, Germany) was used. The mobile phases consisted in 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The following linear gradient of solvent B (min/%B): (0/2), (4/2), (6/50), (7/50), (9/2), (12/2) was applied. The flow rate was set to 250 μL/min and the injection volume was 10 μL. In order to skip the backside effects of the use an ion pairing agent such as perfluoropentanoic acid, a thermostated (30 °C) Synergi-Hydro column (150 × 2.0 mm, 4.0 μm, Phenomenex, Torrance, CA) was used and the analytes were detected through a heated electrospray interface (HESI) operating in the positive mode and scanning the ions in the m/z range of 60–400. The resolving power was set to 75,000 full width at half maximum (FWHM, m/z 200) resulting in a scan time of 1 s. The automatic gain control was used in balanced mode (1 × 10⁶ ions); maximum injection time was 20 ms. The interface parameters were as follows: spray voltage 3.7 kV, capillary voltage 12 V, skimmer voltage 17 V, capillary temperature 275 °C, heater temperature 200 °C, sheath gas flow 30 and auxiliary gas flow 9 arbitrary units. All the above-mentioned parameters were optimized by infusing directly into the HESI source 10 μg/mL of both APs at a flow rate of 10 μL/min. Before intraday analysis the instrument was externally calibrated by infusion of a solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v). The exact mass of diisooctyl
phthalate ([M + H]+: 391.28429) was used as lock mass for the recalibration of the instrument during the analysis. The mass tolerance ranges were fixed to 3 ppm. Target analytes were quantified using the external standard technique: 10 mg of each compound was accurately dissolved in 1 mL of water and a calibration curve was built in the range 10 ng/mL – 10000 ng/mL according to the identification of the limit of detection (LOD) and quantification (LOQ). The carryover effects were monitored by injecting after each run a solution of acetonitrile in water (70%. v/v). The recovery test was performed in order to verify the efficiency of the amino acids and APs extraction procedure: two emulsified control samples that consisted in the same amino acids solutions described above without the presence of the two amino acids, were spiked with 50 ng/mL, 500 ng/mL and 5000 ng/mL of a mixture of both Amadori compounds, Leu and Phe (ion currents associated to specific m/z ratio: 294.15473, 328.13908, 132.10191, 166.08626, respectively) and the values of the area counts were plotted against the area counts of specific calibration curves.

**D-Glucose analysis**

D-Glucose was monitored by using a glucose oxidase/peroxidase assay kit (GAGO-20, Sigma Aldrich, St. Louis, MO). Each sample was diluted 50 times in water and 1 mL was measured in a 10 mL volumetric flask; 2 mL of the assay reagent, that consisted in a mixture of glucose oxidase/peroxidase (500 units and 100 purpurogallin units, respectively) and o-dianisidine 0.1 mg/mL, were added. The flasks were incubated at 37 °C in a light protected incubator. After 30 min the brown o-dianisidine was converted into pink colored oxidized o-dianisidine by adding 2 mL of a solution 12 N of sulphuric acid. The absorbance of this last solution was measured at 540 nm by using a T92+ UV double beam spectrophotometer (PG Instruments, Leicester UK). A D-glucose calibration curve was built in the range 20 – 100 µg/mL by dissolving 1 mg of D-glucose in 1 mL of 0.1% benzoic acid. The calibration curve was performed three times in the same day and three times for three subsequent days for reproducibility and repeatability.
Chapter 5

Statistical analysis

The analyses were performed in quadruplicate by monitoring the target analytes, glucose, Leu, Phe and the two Amadori compounds in four independent O/W emulsions and control systems. The results were expressed as mM for glucose and amino acids or μM for Fru-Phe and Fru-Leu. The kinetic profile were monitored by using Matlab R2009b (Mathworks, Natick, MA).

Results and discussion

Process setup

A set of preliminary experiments was performed to identify the most suitable formulation and processing conditions to get a heat stable emulsion system (data not shown). These experiments ended up in a system with the continuous phases consisting of mixtures of glucose and Leu or Phe, and Tween 20 (1%, final concentration in emulsion) while the apolar lipid phase consisted in glyceryl trioctanoate (10%, final concentration in emulsion). These last two compounds were selected according to their physicochemical properties: glyceryl trioctanoate was used for its low melting point (10 °C) and hence its liquid state at room temperature.\(^{271}\) By using glyceryl trioctanoate, it was possible to simultaneously avoid the crystallization of the lipidic phase, and prevent any formation of hydroperoxide and carbonyls via hydrocarbon cleavage.\(^{263, 272}\) Trioctanoate is in any case enough hydrophobic to have high affinity for the side chain of Leu and in a certain extent for Phe. Tween 20 was used as an emulsifier, as we aimed at forming physically metastable emulsions.\(^{273, 274}\)

Physicochemical characterization of emulsions

The production process was designed in order to obtain typical droplets sizes commonly found in homogenized food emulsions.\(^{256, 275, 276}\) The physicochemical properties of the particles are reported in Table 5.1. For both freshly prepared emulsion samples, the static light scattering analysis revealed a single narrow peak. The surface weighed mean diameter (\(d_{32}\)) ranged 0.144 ± 0.003 to 0.157 ± 0.006 μm and from 0.138 ± 0.002 to 0.171 ± 0.002 μm for the emulsions containing Leu and Phe, respectively. Interestingly, the particle sizes remained constant over the timescale of the experiment (i.e., for 24 h at room temperature, data not shown) and only slight differences could be ascribed to the thermal treatment (Table 5.1).\(^{272, 277}\)
The interfacial area per mass unit of dispersed phase, or specific surface area ranged from 40.14 ± 0.02 to 43.84 ± 0.05 m²/g of oil for Phe system while for Leu system they ranged from 36.48 ± 0.01 to 45.62 ± 0.04 m²/g of oil. There was no substantial difference in the mean diameter of emulsions containing Leu or Phe, which implies that the chemical characteristics of both tested amino acids and the presence of reducing carbonyls did not influence the physical properties and stability of the emulsions. These results are in line with those previously obtained by other authors.257 278

Table 5.1:  Measured physicochemical properties of the O/W systems. The specific surface area A, or interfacial area per mass unit of dispersed phase was calculated according to the equation (1). Dispersant refractive index 1.33; particle refractive index 1.45 (glyceryl trioctanoate). Surface weighted d [3, 2]; d [4, 3], volume weighted.

<table>
<thead>
<tr>
<th>Compound</th>
<th>d [3, 2]</th>
<th>d [4, 3]</th>
<th>Span</th>
<th>A (m²/g oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu O/W T0</td>
<td>0.146 ± 0.001</td>
<td>0.183 ± 0.002</td>
<td>1.226 ± 0.002</td>
<td>43.38 ± 0.04</td>
</tr>
<tr>
<td>Leu O/W T2</td>
<td>0.144 ± 0.001</td>
<td>0.181 ± 0.002</td>
<td>1.264 ± 0.001</td>
<td>43.84 ± 0.05</td>
</tr>
<tr>
<td>Leu O/W T4</td>
<td>0.152 ± 0.001</td>
<td>0.192 ± 0.001</td>
<td>1.329 ± 0.001</td>
<td>41.58 ± 0.09</td>
</tr>
<tr>
<td>Leu O/W T6</td>
<td>0.157 ± 0.006</td>
<td>0.199 ± 0.002</td>
<td>1.365 ± 0.002</td>
<td>40.34 ± 0.04</td>
</tr>
<tr>
<td>Leu O/W T8</td>
<td>0.157 ± 0.001</td>
<td>0.200 ± 0.001</td>
<td>1.374 ± 0.001</td>
<td>40.14 ± 0.02</td>
</tr>
<tr>
<td>Phe O/W T0</td>
<td>0.171 ± 0.002</td>
<td>0.220 ± 0.001</td>
<td>1.476 ± 0.002</td>
<td>36.98 ± 0.01</td>
</tr>
<tr>
<td>Phe O/W T2</td>
<td>0.157 ± 0.002</td>
<td>0.203 ± 0.001</td>
<td>1.466 ± 0.001</td>
<td>40.28 ± 0.04</td>
</tr>
<tr>
<td>Phe O/W T4</td>
<td>0.145 ± 0.001</td>
<td>0.187 ± 0.001</td>
<td>1.332 ± 0.001</td>
<td>43.50 ± 0.01</td>
</tr>
<tr>
<td>Phe O/W T6</td>
<td>0.160 ± 0.002</td>
<td>0.218 ± 0.002</td>
<td>1.651 ± 0.002</td>
<td>39.38 ± 0.04</td>
</tr>
<tr>
<td>Phe O/W T8</td>
<td>0.138 ± 0.002</td>
<td>0.191 ± 0.001</td>
<td>1.574 ± 0.001</td>
<td>45.62 ± 0.04</td>
</tr>
</tbody>
</table>

The overall morphology of the emulsion systems was investigated using optical microscopy (Figure 5.2). Emulsified systems were imaged at the end of each point of the thermal treatment (0, 2, 4, 6 and 8 min at 131°C). Results revealed that only slight increase in droplet size occurred over the thermal treatment and no flocculated droplets could be observed.279 This can be explained by the strong stabilizing effect of Tween 20 by steric interactions, which is not supposed to be affected by increased temperature.257 The stability of the emulsion at time/temperature conditions commonly adopted for food sterilization paved the way for the characterization of the chemical modifications occurring during thermal treatments.
Figure 5.2: Optical microscopy images of emulsion samples (10% glyceryl trioctanoate w/w, pH of the aqueous phase 6.8) before and after thermal treatment (131 °C, 8 min). The emulsion remained stable also after the thermal treatment (see table 5.1).

Amadori compounds formation by HRMS analysis

The detection of APs in the emulsified system required the adaptation of a HRMS procedure recently developed in our laboratory. The summary of analytical performances obtained on the emulsified systems is reported in Table 5.2. The use of a polar end-capped column and the specific chemical nature of these amino acids (i.e. the presence of an aromatic group and an aliphatic side chain), allowed to skip the use of perfluoropentanoic acid. The condition of ions detection, as well as the presence of a recalibrating agent allowed to reduce the mass error up to 0.8, 1.2, 1.3 and 1.0 ppm for Leu, Phe, Fru-Leu and Fru-Phe, respectively. The LOD and LOQ were 0.5, 0.5, 1 and 1 ng/mL, while the limit of detection was 2, 2, 5 and 5 for Leu, Phe and their APs, respectively. The coefficient of determination, $r^2$, was always higher than 0.992 for both intraday and interday assays. The recovery ranged from 93 to 99 % for all the analytes.
Figure 5.2: Optical microscopy images of emulsion samples (10% glyceryl trioctanoate w/w, pH of the aqueous phase 6.8) before and after thermal treatment (131 °C, 8 min). The emulsion remained stable also after the thermal treatment (see Table 5.1).

Amadori compounds formation by HRMS analysis. The detection of APs in the emulsified system required the adaptation of a HRMS procedure recently developed in our laboratory. The summary of analytical performances obtained on the emulsified systems is reported in Table 5.2.

The condition of ions detection, as well as the presence of a recalibrating agent allowed to reduce the mass error up to 0.8, 1.2, 1.3 and 1.0 ppm for Leu, Phe, Fru-Leu and Fru-Phe, respectively. The LOD and LOQ were 0.5, 0.5, 1 and 1 ng/mL, while the limit of detection was 2, 2, 5 and 5 for Leu, Phe and their APs, respectively. The coefficient of determination, $r^2$, was always higher than 0.992 for both intraday and interday assays. The recovery ranged from 93 to 99% for all the analytes.

Table 5.2: Measured HRMS analytical performances. The mass accuracy was calculated dividing the mass error (i.e.: the difference between the theoretical mass and the experimental mass) by the theoretical mass. The results were reported in ppm by multiplying one thousand.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exact mass (m/z)</th>
<th>Mass Accuracy (ppm)</th>
<th>Recovery %</th>
<th>LOD (ppb)</th>
<th>LOQ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>132.10191</td>
<td>0.8</td>
<td>93</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Fru-Leu</td>
<td>294.15473</td>
<td>1.3</td>
<td>94</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Phe</td>
<td>166.08626</td>
<td>1.2</td>
<td>99</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Fru-Phe</td>
<td>328.13908</td>
<td>1.0</td>
<td>94</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

In Figure 5.3, the formation of Fru-Phe and Fru-Ile along with reactants’ degradation (glucose, Leu and Phe), during thermal treatment was reported. The concentration of the three reactants (glucose, Phe and Leu) in aqueous solutions and in emulsions decreased with increasing the thermal treatment time. No substantial differences were observed between the aqueous phase system and the emulsion system as far as the decrease of glucose and Phe concentration. On the contrary the concentration of Leu after 6 and 8 min of the thermal treatment was much higher in the emulsion system than in the control aqueous system, suggesting that Leu may have been separated from the glucose present in the aqueous phase, for instance by a preferred location at the interface of the oil droplets.

Figure 5.3: Kinetic profiles of glucose, amino acids and respective APs concentrations. Red dots represents the O/W microemulsion systems, while the blue dots report the control aqueous samples.
These results were nicely confirmed by the data about the APs formation. Independently of the presence of oil droplets, the maximum concentration of the two APs was observed after 6 min: $2.053 \pm 0.035$ μM and $2.093 \pm 0.123$ μM for Fru-Phe in control aqueous solution and emulsion, respectively; $0.680 \pm 0.037$ μM and $1.229 \pm 0.025$ μM for Fru-Leu in control system and emulsion system, respectively. At the end of the thermal treatment, while the concentration of Fru-Leu rapidly decreased up to $0.507 \pm 0.011$ μM and $0.948 \pm 0.011$ μM respectively, the concentration of Fru-Phe remained almost constant: $2.198 \pm 0.069$ μM and $1.984 \pm 0.033$ μM. Focusing on the systems with Leu, we observed a reduction of the formation of Fru-Leu up to 41%, 21%, 45% and 47%, after 2, 4, 6 and 8 min, respectively, compared to the control aqueous system. The results for Phe were quite as no significant effects of the O/W emulsion system can be observed, compared to the control aqueous system. After 2, 4 and 8 min data showed an slight increase in the concentration of Fru-Phe in the emulsion system compared to the aqueous solution (20%, 38% and 10%, respectively), while after 6 min it was almost the same in the two systems with only 2% reduction.

Control data before thermal treatment confirmed the stability of the developed emulsified model system: as expected almost no APs was detectable and the concentration of the reactants (glucose Leu and Phe) was decreased only by 5% during the emulsion preparation. This minor loss may be tentatively explained by a partial degradation and isomerization of glucose and with a partial oxidation of the amino acids during the initial phase of the emulsion preparation.219

Reactants location and Maillard reaction

The presumed reaction pathways leading to the formation of the two APs in presence and in absence of oil droplets are highlighted in Figure 5.4. In both systems it can be assumed that the initial step is the formation of a glycosylamine that under the described reaction conditions rearranges to form the corresponding APs, i.e., Fru-Phe and Fru-Leu. Data showed that in the emulsion system the formation of Fru-Leu was reduced up to 50%, while Fru-Phe seemed to be hardly influenced by the presence of oil droplets.

These results can be tentatively explained with the two different partition coefficients of the amino acids Phe and Leu which are $0.235 \pm 0.277$ and $0.799 \pm 0.275$, respectively.282 In fact, to explain the different reactivity of Leu with glucose in aqueous versus emulsion systems, the distribution of amino acids between the different regions of the emulsion should be considered.
The system is considered at thermodynamic equilibrium and the reactants’ location is controlled by their relative solubility in each region. The distribution of reactants (and of any other component, such as Tween and oil) between regions in emulsions is rapid at the time scale of the chemical reactions. The hydrophobic portion of Leu (an aliphatic isobutyl chain) would be oriented into the oil region, but its amino and carbonyl groups would be anchored within the interfacial region. Because of the presence of ionizable groups, we assume that the concentration of both amino acids in the oil droplet core is negligible, and reaction occurs only in the aqueous phase or in the interfacial region. Besides the oil-water interface, Tween 20 micelles can also constitute a favorable environment for the two amino acid location.

![Diagram of Amadori products formation in emulsified systems](image)

**Figure 5.4:** Mechanism of Fru-Leu and Fru-Phe formation in aqueous media, in blue, and in presence of O/W emulsion, highlighted in yellow. Leu aliphatic side chain drag this amino acid close to the lipid moiety decreasing the reactivity of its amino group with glucose (dotted line).

The presumed location of Leu at the oil-water interface can explain why the amino group of Leu was less prone to the formation of APs, as revealed by the kinetic formation profile. On the opposite, the reactivity of an amino acid with a lower logP, such as Phe, was less affected by the presence of oil droplets, as similar concentrations were observed in control aqueous and emulsion systems. In the model hereby proposed, the affinity of Leu for environments with a lower polarity than water, such as the oil-water interface or surfactant micelles, reduced the reactivity of this amino acid, resulting in the reduction of Fru-Leu formation.
These results are in agreement with previous findings on the microreactor activity of microemulsions. Lutz and co-workers demonstrated that in U-type microemulsion, where the lipid phase consisted in butanol, propylene glycol and R(+)‐limonene, the reaction rates can be controlled by the microemulsion type. The mobility of the reactants and water activity affected by the microemulsion interface curvature and the composition of the system at the end of thermal treatment can be well explained by the microemulsion ability to control the reaction rates. Vauthey and co-workers reported that structured fluids, such as association colloids and isotropic fluids with unsaturated monoglycerides, strongly influence the thermal generation of volatiles, where the reactants can be trapped at interface. Locally high concentrations at the interface strongly accelerate bimolecular reactions in presence of polar amino acids and reactants, such as cysteine, furfural as well as diacetyl and other dicarbonyls. The use of hydrophilic amino acids, polar basic or neutral amino acids with a lower partition coefficient can hypothetically promote the migration of the reactants in the aqueous layer. In a closed system the presence of microemulsion reduced the water activity increasing the reaction rate and the extent of the MR. The O/W reaction medium in presence of extremely polar compounds enhances the reaction rates in comparison to the water-based reaction.

The data obtained with the two amino acids used in this model systems provide a preliminary overview on the reaction kinetics of Amadori compounds formation in O/W emulsion systems. As described above, according to the partition coefficient of the amino acids, it seems possible to alter the different reactants’ location and optimize several strategies to control not only the final extent of the Amadori compounds formation, but also the final direction of the MR, according to the chemistry of amino acids.

**Conclusions**

In this work a reliable and reproducible emulsion system stable to the time temperature conditions commonly occurring during food thermal treatment has been developed and combined with a robust HRMS procedure to quantify the neo formed compounds generated by the thermal treatments. The work focuses exclusively on Amadori compounds formation in dispersed systems, while volatiles and end-products formation, as well as the interplay between lipid oxidation and MR will be the subject of a forthcoming work. The evaluation of the various reaction mechanisms has to consider the presence of emulsions that can deeply influence the yield of compounds.
formed. Reactants location is a relevant aspect for the APs formation having the potentiality to modulate the reaction pathway according to the kind of the amino acids and to the presence of O/W systems.
Chapter 6

General discussion
Introduction

Under the name of Maillard reaction (MR) an intricate network of reactions takes place. The nature and concentrations of the products of these reactions, indicated as Maillard reaction products (MRPs), determine the final quality of thermally processed foods. The formation of both undesired and desired molecules is the central dichotomy in the Maillard cascade where the nodal point is represented by the Amadori products (APs) and its analogous Heyns products (HPs), the first stable compounds. The final direction of the reaction is mediated by the fragmentation, degradation and conversion of the APs and HPs: color, aroma, taste, flavors, texture as well as the formation of potentially toxic molecules and off-flavors are the main attributes regulated by the schema presented in Figure 6.1. The control of the MR can be considered as a smart tool to achieve the desired food quality, while MRPs quantification allows to monitor food processing with direct implications in the food industries and consumers acceptance.

Figure 6.1: The Hodge schema is proposed by combining the color development in some foods, the stage of the MR. The melanoidins structure was adapted from Moreira and co-workers.286
Pivotal papers have identified the most significant markers of the MR. In Figure 6.2, the arrows indicate the pathways leading to different molecules with direct implication in the MR. In milk and dairy products, the quantification of Ne-(1-Deoxy-D-fructos-1-yl)-L-lysine (fructose-lysine), and its conversion product, Ne-(2-furoylmethyl)-L-lysine (furosine), Ne-(Carboxymethyl)-L-lysine (CML), Ne-(Carboxyethyl)-L-lysine (CEL), pentosidine, pyrraline, lysino-alanine and 5-hydroxymethylfurfural (HMF), along with volatiles and off-flavor are relevant to determine the final quality. In carbohydrate rich food, treated at high temperature, i.e. coffee, bakery products and potatoes along with the above listed markers, the determination of acrylamide and HMF become dominant not only for the evaluation of the thermal loading, but also for safety issues. 

Figure 6.2: Overview of the MR markers studied, different arrows condensate different reaction pathways arising from the APs.

The preservation of attributes such as color, texture and aroma, the reduction of several potentially toxic compounds and the possibility to orientate the Maillard reaction pathways according to the precursors and intermediates concur to combine the risk-oriented approach and the quality-oriented approach.
In practice, food scientists are called to define coherent strategies able to circumvent the formation of *bad and ugly MR molecules* (i.e. the potentially toxic molecules quoting Thomas Henle) and to promote the formation of *good MR molecules*. Once the key control points and end-points are defined, it is possible to setup the optimal mitigation strategies: outcomes definition (i.e. the reduction of acrylamide), then the optimization of the strategy (i.e. the use of enzyme), finally the target molecule, or class of molecules are investigated.

The tuning of time and temperature couple is the most common approach to modulate the thermal load in the food industry. With it, the external control of the MR is obtained to limit the formation of undesired outcomes keeping as much as possible the desired quality features. Specifically, the parallel reduction of acrylamide formation and the formation of yellow/brown color in potato model system is one of the examples. De Vleeschouwer and co-workers demonstrated that acrylamide formation is slightly more temperature-dependent than color formation. This implies that reducing temperature will reduce acrylamide formation more than color formation.

With the progression of the knowledge on the nonenzymic browning it is possible to propose more sophisticated strategies to control the reaction limiting the formation of the undesired compounds without affecting the development of the desired features too much. The subtraction of reactants from the reaction mixture by using different encapsulation techniques, the masking of aliphatic amino acids in microemulsion systems, the use of polyphenols compounds from olive oil mill wastewaters able to interfere with dicarbonyls and free amino groups, the use of an enzymatic technique able to deglycate Amadori products are the four alternative strategies presented here, aiming at fine tuning of the MR along with the MRPs formation. This can be defined as an holistic approach: the mitigation strategies involve each step of the MR, resulting in the reduction of undesired molecules without influencing the formation of desired molecules. The individuation of the precursors and the reactants followed by the mechanistic evaluation of key intermediates of the Maillard reaction is defined as the paradigm for the setup of the mitigation strategies where the ultimate goal is the production of foods having a better quality. Several alternatives are possible and a general overview of the different strategies is offered in Table 6.1. The strategies range from the reduction of the thermal loading to the use of kinetic modeling and novel tools evaluated in this thesis. Pros and cons are summarized in order to obtain a sketch of the possible process optimization.
In practice, food scientists are called to define coherent strategies able to circumvent the possible process optimization. Alternatives are possible and a general overview of the different strategies is offered in strategies where the ultimate goal is the production of foods having a better quality. Several defined as an holistic approach: the mitigation strategies involve each step of the MR, resulting in aliphatic amino acids in microemulsion systems, the use of polyphenols compounds from olive oil compounds without affecting the development of the desired features too much. The subtraction more sophisticated strategies to control the reaction limiting the formation of the undesired outcomes keeping as much as possible the desired quality features.

The tuning of time and temperature couple is the most common approach to modulate the finally the target molecule, or class of molecules are investigated. Specifically, the parallel reduction of acrylamide formation and the formation of yellow/brown color formation of undesired outcomes keeping as much as possible the desired quality features. The volatiles can be influenced, it depends on the sugars present and their reaction rates. Color and flavor cannot be formed. Microorganisms, digestibility, antioxidants release, texture are negatively influenced. Safety issues. Changes in volatiles formation. Color formation, water removal, texture. Application to viscous and semisolid samples, internal pressure. Large scale application should be verified. Principle of Le Chatelier: MR must be taken into account, backside reaction due to protein denaturation, removal of typical flavors. Can be applicable to fruits.

### Table 6.1: Overview of pros and cons of the control strategies of the MR.

<table>
<thead>
<tr>
<th>Route</th>
<th>Strategy</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal load</td>
<td>Refrigeration</td>
<td>Most foods will not brown below -10° C</td>
<td>The volatiles can be influenced, it depends on the sugars present and their reaction rates.</td>
</tr>
<tr>
<td>Time/Temperature</td>
<td>MR is temperature dependent, all the pathways and potentially toxic molecules are reduced.</td>
<td>Color and flavor cannot be formed. Microorganisms, digestibility, antioxidants release, texture are negatively influenced. Safety issues.</td>
<td></td>
</tr>
<tr>
<td>Microwave</td>
<td>No specific changes in proteins; reduction of HMF</td>
<td>Changes in volatiles formation.</td>
<td></td>
</tr>
<tr>
<td>Ohmic heating</td>
<td>Reduction in the thermal loading.</td>
<td>Color formation, water removal, texture. Application to viscous and semisolid samples, internal pressure.</td>
<td></td>
</tr>
<tr>
<td>Radiofrequencies</td>
<td>Reduction in the thermal loading</td>
<td>Large scale application should be verified.</td>
<td></td>
</tr>
<tr>
<td>High hydrostatic pressure</td>
<td>Minimally processed foods, reduction of thermal induced toxicants, fresh-like attributes</td>
<td>Principle of Le Chatelier: MR must be taken into account, backside reaction due to protein denaturation, removal of typical flavors.</td>
<td></td>
</tr>
<tr>
<td>Pulsed electric field</td>
<td>Non thermal treatment, reduction of HMF and intensive color formation</td>
<td>Can be applicable to fruits.</td>
<td></td>
</tr>
<tr>
<td>Reactants</td>
<td>Bivalent/monovalent cations</td>
<td>Reduction of $a_w$, increase in the reaction rates, reduction of acrylamide formation</td>
<td>Formation of HMF, according to the compounds used the pH can be influenced.</td>
</tr>
<tr>
<td>Addition of amino acids</td>
<td>Reduction and elimination of acrylamide; Removal of carbonyls, sulphur can be removed by heating</td>
<td>Increase in the browning, volatiles are influenced.</td>
<td></td>
</tr>
<tr>
<td>Sulphur dioxide</td>
<td></td>
<td>Detected by taste, regulatory and legal aspects. Safety issues.</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Promotes the reduction of the pH, favoring other mechanisms</td>
<td>Increasing the concentration the browning is due to the oxidation of ascorbic acid. Direct ascorbylation of lysine and amino groups</td>
<td></td>
</tr>
<tr>
<td>Enzymes</td>
<td>Glyoxalases</td>
<td>Conversion of α-oxoaldehydes into α-hydroxyacids</td>
<td>Cellular mechanism, application to foods?</td>
</tr>
<tr>
<td>Fructosamine-3-kinase</td>
<td>Phosphorylation of APs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparaginase</td>
<td>Oxidation of asparagine into aspartic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agronomy</td>
<td>Biochemistry</td>
<td>Reduction in the asparagine content, use of reducing sugars</td>
<td>Color and flavor can be influenced.</td>
</tr>
<tr>
<td>Kinetic Modeling</td>
<td>Multiresponse</td>
<td>Prediction of end markers formation</td>
<td>Influence of the substrate. Large scale applications.</td>
</tr>
<tr>
<td>Novel approaches</td>
<td>Encapsulation</td>
<td>Sequestrating reactants (NaCl, ascorbic acid, iron, PUFA) without affecting sensorial properties</td>
<td>Hydrophobic coatings can chelate nutrients, reduction of $a_w$. Large scale application, effects on off-flavors, formation of 1-deoxyglucosone</td>
</tr>
<tr>
<td>Faox I and II</td>
<td>Reduction of MRPs, beside the reduction of free APs</td>
<td>Acacia fiber and maltodextrins can block water molecules and influence the MR pH, reaction rates negatively influenced according to the partition coefficient of amino acids</td>
<td></td>
</tr>
<tr>
<td>Spray dried OMWW</td>
<td>Phenylethanoloids reduce dicarbonyls, hydroxycarbonyls, off-flavor, MRPs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microemulsions</td>
<td>Modulate precursors location, control the reaction rates, modulate flavor formation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


**Snapshot of thesis main achievements**

This thesis is focused on four novel strategies in the control of the MR, each of them is able to interfere in the chemical pathways by tuning the location of the key precursors and the formation of both intermediates and end-markers.

- **Reactants encapsulation**

In Chapter 2, a new perspective for the encapsulation technique was illustrated. Encapsulation refers to the technology of enclose solid, liquid and gaseous material in small capsules that release their contents at controlled rates over prolonged periods of time or under specific process conditions. Encapsulation strategies have found wide application in food industries and recently some papers have introduced a new concept: not only the protection and the delivery of bioactive and functional molecules, but also the subtraction of some reactants that, although present at relatively low concentration, are able to influence the extent of Maillard reaction. An overview of the key reaction pathways leading to the formation of Maillard reaction end-products was proposed focusing on the reactants with direct implication in determining the final extent of the reaction. Firstly, it was observed that incorporating encapsulated PUFA in bread was linked to the decrease of HMF and acrylamide, considering that carbonyls arising from the thermoxidation of PUFA during baking, can promote the conversion of asparagine into acrylamide. Secondly, the encapsulation of NaCl was an effective strategy to control the formation of some MRPs, such as HMF: the pyrolysis of glucose and the dehydration of fructofuranosyl cation can be efficiently prevented by three different hydrophobic coatings. The key point was the thermal resistance of the wall material: the higher the melting point the more pronounced the reduction of HMF formation. Interestingly enough, this procedure did not modify the sensorial properties of the cookies as NaCl was released before the end of the cooking process. Thirdly, the encapsulation of NaCl is potentially suitable for many other food products and for the control of other reaction such as the nucleophilic attack of chloride ions for the formation of 3-monochloropropane-1,2-diol (3-MCPD). This molecule belongs to a group of well-known process-induced contaminants derived from mono- and di-chlorinated glycerols; even if its occurrence can rely outside the MR, the encapsulation of sodium chloride have the potential to limit or suppress the $S_n2$ nucleophilic attack by chloride ions. Finally, evidences suggested that other Maillard reactants, such as bivalent cations, iron and ascorbic acid can be encapsulated. Iron and bivalent cations can chelate and coordinate water molecules. In presence of iron ions the rate of browning and the formation of
dicarbonyl compounds are influenced by the oxidation reactions. The relationship between ferric ions and PUFA peroxidation and degradation of ascorbic acid was verified and the encapsulation of both ascorbic acid and iron can promote the reduction of the concentration of dicarboxyls involved in the formation of Ne-fructose-lysine, CML and CEL via Namiki pathway. Moreover, ascorbic acid promoted the reduction of lysine availability via direct ascorbylation of the amino group or by leading to the formation of furfural and other Maillard intermediates. Preliminary evidences highlighted that the use of stearic acid coating significantly reduces the Maillard-type browning reactions of ascorbic acid in milk infant formula via the formation of dehydroascorbic acid and other degradation products.

The introduction of encapsulated ingredients in foods also poses some technical drawbacks. Specific strategies should be developed to dissolve the capsules in liquid foods in order to obtain an homogeneous dispersion therefore the use of hydrophobic coating can be the main drawback for beverages. On the other hand, in bakery products the lipophilic capsules can be accurately dispersed in the dough in order to prevent the aggregation of particles with negative outcomes.

However, the costs of encapsulated ingredients may represent a hurdle when their concentration in use is significant.

- Use of deglycating enzymes

In 1997, Monnier and co-worker isolated two fructosamine oxidase enzymes, Faox I and II from fungi. Both enzymes are able to use Amadori compounds as substrate, even if the X-ray structure revealed that the activity toward peptides varied according to the sizes and to the steric hindrance of the glycated substrate lysine residues. During the last ten years Faox enzymes have been extensively used by our group and the main evidences are reported in Table 6.2 along with a general overview on the potential application of the enzyme in tomatoes derived products and low lactose milk.
In Chapter 3 of my thesis, the use of Faox I and II in a complex system such as low lactose milk and in model systems consisting in β–lactoglobulin and glucose was evaluated as tool to control the formation of glycated peptides as well as non-enzymatic glycation markers. CML and bound 5-hydroxymethylfurfural (b-HMF) were monitored by LC-MS/MS and UV-Vis, while glycated β–lactoglobulin peptides were characterized by MALDI-TOF analysis. The results showed a reduction in glycated peptides CML and b-HMF in all systems investigated. Specifically, the overall reduction ranged from 42.0 % to 94.4 % for b-HMF, while the reduction of CML ranged from 24.4% to 58.3%. Along with clear evidences in the deglycation activity, there are several aspects that need to be further investigated. The mechanism of action implied a local unfolding of the enzyme prior the carbonyls attachments, while due to inaccessibility of the already glycated site no clear effect can be drawn, as highlighted in Figure 3. The presence of the enzyme before the glycation of the lysine promotes the reduction of APs formation.
The control of the formation of the Amadori compounds in free and bound lysine residues resulted in the reduction of the advanced stage products. The effects of the storage on bound MRPs paved the way for the study of both free amino acids, Amadori compounds, aroma volatile and off flavor. A relatively high percentage of chemical modifications in foods occurs on the free amino acids and the control of free Amadori compounds formation requires specific strategies: the volatiles and off-flavor formation during the storage of free amino acids rich foods, such as tomato or orange juice is one of the examples. Specifically, when Faox is added to tomato juice before thermal treatment, a significant reduction of MR induced volatile compounds, such as furfural and 2-isobutylthiazole has been found in tomato juice heated at 90°C for 10 min (Yimin Chen, Master thesis 2015). Faox enzymes can switch the reaction pathways by limiting the reducing sugars attachments on α-amino group. The final results involve the overall reduction of C-2 and C-4 fragmentation, whose direct consequence is the increase in α-dicarbonyls, α-hydroxycarbonyls, acetic acid via amine induced β-cleavage.

Figure 6.3: Post addition of Faox (top panel) and pre-glycation addition of the enzyme (bottom panel) and formation of APs.
All in all the data on the action of this class of de-glycating enzymes in foods it suggests that the prevention of molecular modifications usually taking place during processing could result in the achievement of food freshness attributes.

- **Use of polyphenols from olive oil mill waste water (OMWW) to prevent MR off-flavor formation**

The effect of polyphenols on carbonyl trapping activity has been intensively studied. Peterson and Ho’s group showed that the reaction between α-dicarbonyls such as glyoxal and methylglyoxal and polyphenols, i.e. catechins promotes the formation of a covalent linkage between the C1 position of the methylglyoxal and either the C6 or the C8 position of the EC A ring, presumably generated by hydroxyalkylation and aromatic substitution reactions. 20, 223 These evidences represent the background and the rationale for the use of polyphenols in the control of the MR. In **Chapter 4**, along with the evaluation of the chemical insights beside the reduction of flavor compounds and MRPs, a new strategy was introduced: the use of a byproduct, such as olive oil mill wastewaters (OMWW) as a source of phenylethanoids was dissolved in milk prior the thermal treatment. The OMWW–based ingredient was obtained by spray drying with maltodextrin and acacia fiber coating and upon the quantification of tyrosol, 3-hydroxytyrosol and verbascoside, the effects were evaluated in lab scale UHT milk by monitoring off-flavor (methional and 2-acetyl-2-thiazoline) α-dicarbonyls (methylglyoxal, glyoxal, 2,3-butanedione and 3-deoxyglucose), α-hydroxycarbonyls (glycoaldehyde, acetoin, and acetol), one of the main precursors (total lysine) and bound Maillard reaction products (furosine and CML). Results showed significative reduction of all the compounds listed above in presence of the OMWW ingredient, with acetol and 3-deoxyglucose, as the only exception, while total lysine remained unaltered. The chemical aspects beside the use of spray-dried OMWW highlighted that a trisubstituted phenolic ring with an o-dihydroxy function can react with dicarbonyls by hydroxyalkylation and aromatic substitution reaction, thus showing trapping activity toward methylglyoxal and glyoxal. In the presence of pro-oxidant agents the phenolic ring can be oxidized to quinone, whose reaction with side chain of lysine and other amino group can lead to the formation of iminoquinone and iminophenol via Schiff bases. An overview of the two mechanisms hypothesized is presented in **Figure 6.4**.
Some studies had already reported that the use of polyphenols is efficient strategy for the reduction of dicarbonyls, off flavor and hydroxycarbonyls in UHT milk. The findings of this thesis suggested that in the future also other optimization tools for the application of phenolic mixtures as a preprocessing treatment will have to be taken into consideration. They involve the use of response surface methodology and of specific carriers and coatings able to guarantee the accurate dispersion and the physicochemical stability of the phenolic mixtures in foods. Preliminary, non-published, results highlighted that despite secoiridoids are able to control the formation of MRPs in biscuits the presence of maltodextrin and acacia fiber in the dough can sequestrate water molecule promoting the local reduction of the water activity creating the ideal environment for the formation of HMF and acrylamide. Once again, this observation illuminated the double face of the modifications during thermal treatments: the control of the MR in food by using polyphenols should consider dose/response activity and the appropriate coating able to prevent backside effects.

- **MR development in microemulsion systems and influence of reactants location on Amadori products formation**
Since the crucial paper of John Hodge was published in 1953, the formation of the Amadori products has been extensively studied in aqueous and dry model systems.\textsuperscript{2} In Chapter 5, a solution consisting in glyceryl trioctanoate, Tween and a mixture of leucine or phenylalanine and glucose was microemulsified by an high pressure homogenizer. The droplets size in the microemulsion system ranged from 0.13 to 0.20 µm while the formation of the Amadori products from phenylalanine and leucine (N-(1-deoxy-D-fructos-1-yl)-L-phenylalanine, Fru-Phe and N-(1-deoxy-D-fructos-1-yl)-L-leucine Fru-Leu, respectively) was monitored in emulsion and aqueous system. High resolution mass spectrometry results showed that Fru-Phe was not influenced by the presence of microemulsion, while in the dispersed droplets the formation of Fru-Leu was reduced up to 50%. Data suggested that the partition coefficient of the two amino acids (0.80 and 0.23 for leucine and phenylalanine, respectively) plays a key role in the reactants location and in the final extent of the reaction. It has been hypothesized that the amino group of leucine can be masked in the proximity of the lipid phase thus reducing its reactivity towards glucose.

This preliminary study opens a new scenario in the Maillard chemistry: microemulsions directly influence the reactants location and that is one of the crucial parameters in determining the final direction of the reaction. The effects of microemulsion and location have been already evaluated for flavor and aroma formation.\textsuperscript{83} The study of APs formation in microemulsion systems can offer new insights in the chemical mechanisms of end products formation in foods. Moreover, the relationship between lipid droplets, reactant location and desired compounds formation has the potentiality to promote the development of foods, where according to the reactants location and to the MR it is possible to modulate their final quality.

**Impact of the results on academy and industry**

The beneficial aspects of thermal processing include several benefits that encompass the inactivation of food-borne pathogens and natural toxins, the formation of desired texture, color and flavor, the release of antioxidants and other functional molecules and the improvements of digestibility.\textsuperscript{28} These effects are direct consequences of an interaction between food matrices and the thermal process applied, that results in the formation of heat induced toxicants. In this frame the Maillard reaction is a crucial aspect and its tuning, along with the control of lipid oxidation, is the route to improve food quality. The entangled network of reactions collectively included in the Maillard chemistry cannot be confined to the reaction between amino group and carbonyls from
sugars it is useful to include also lipids, vitamins, antioxidant compounds and other reactants such as bivalent cations, sodium chloride and iron. During the storage and the thermal treatment, food constituents are not inert, they react each other influencing the final quality of foods.

In the last fifteen years, the control of the Maillard reaction has become sometimes synonym of “control of acrylamide formation” and the efforts have been mainly addressed to the reduction of this potentially toxic compounds in carbohydrate rich food.\textsuperscript{15,29} In any case, the \textit{acrylamide issue} represented a reliable example of transferring knowledge from Academy to industry. The study of acrylamide formation, the role of the precursors and the mechanistic insights paved the way for the setup of the reduction strategies and for the feasibility of their use in large scale production. As a consequence the food industries imported, ameliorated and in some situation extensively applied several mitigation strategies that were firstly developed in the research laboratories. They include reduction of the thermal loading, changes in recipes and formulations such as selection of potato and cereal varieties low in acrylamide precursors; addition of proteins, glycine, cysteine and other amino acids which act as competitors towards asparagine, addition of organic acids, acidulants and calcium ions; addition of the enzyme asparaginase.

Even if in this thesis the focus was not only on acrylamide mitigation strategies, two questions still arise when its findings are compared to the research development about acrylamide:

- Is it possible to foresee for the tools proposed in this thesis an application in industry as it happened for acrylamide?
- Which could be the impact of reactants encapsulation, microemulsion, de-glycating enzymes and use of secoiridoids derivatives on food quality?

Only limited answers can be provided at this stage and they will largely depend on the different perspective of Academy and Industry. On the one hand, from the Academic point of view, it is evident that these tools can have a positive impact on food quality.

On the other hand, from the Industrial point of view the answer is far more complicated. In all cases presented here, the pathways to obtain foods of better quality would face some regulatory issues while technological feasibility and scale-up potentiality should be simultaneously considered. As examples, the production of microemulsified systems requires several technological efforts the and large scale industrial adjustments are necessary; Faox enzymes have
to be tested in a large scale production and the effects of thermal treatments in milk should be investigated.

A detailed study of the potential industrial application of the developed strategies is outside the target of my thesis, but there are two aspects that represent the main motivations to the introduction of new foods where the MR is controlled. First, in the last decades, the consumers’ attention to the dietary AGEs outcomes and the carbonyls loading in relationship to chronic disease has increased. As a consequence, the Maillard reaction mitigation strategies along with the prediction of the formation of end-markers such as acrylamide, HMF, furan, CML, CEL and 3-MCPD have found a direct correspondence in the food industry. Second, the Maillard reaction is not only the key for the aroma generation, but also for off-flavor formation and great attention has been reserved for the issue.

**Future recommendation and forthcoming strategies: a) tuning reactants location**

Although the four strategies proposed in this thesis may appear different at first glance, they share two common characteristics: the *location* of reactants and the *interaction* between precursors, intermediates and other molecules or enzyme. The take home message of this thesis can be condensed in three keywords: Maillard, location and interaction. The word *Maillard* is almost obvious, while the other two are not. In **Figure 6.5**, the titles of the articles present in Scopus typing the words “Maillard reaction” are illustrated by a word clouding. The keywords of the articles from 1947 up to 2014 have been collected: the bigger the word in the figure, the higher its frequency in Scopus. Location and interaction have not been studied in relation to Maillard reaction development thus far.
Figure 6.5: Word clouding performed typing in Scopus the word “Maillard reaction” (1947-2015 in the following areas: Chemistry, Agriculture and Biological Sciences, Biochemistry, Genetics, Medicine, Engineering, Chemical Engineering) and taking into account only the keywords of the publications: higher the surface of a single word, higher the frequency of the keyword in Scopus.

The following bullet point list highlights why location and interaction can be so important in the control of the MR and tries to envisage how far we can go from here.

- Encapsulation limits the formation of MRP blocking reactants that play a key role in the development of MR such as sodium chloride, PUFA, iron, ascorbic acid. The reduction of the interaction between these molecules located inside the capsules and MR precursors occurs. The effectiveness of the encapsulation of reactants needs to be tested in aqueous liquid systems where the dispersion of lipidic capsules is the main drawback. The encapsulation of ascorbic acid, iron and PUFA in lipidic coating requires a complete dispersion of the capsules and aggregates and in this case creamy beverages are the ideal substrate. Despite the exciting potentialities there are several gaps limiting the use of this technology in liquids and each food matrix needs to be tested time by time according to the coating material and to the reactants that can be encapsulated to inhibit the reaction. Another point of attention is the relationship between reactants and the pH of the medium. The encapsulation can indirectly control the pH of the mixture and indirectly the extent of the Maillard reaction. The release of some reactants, mainly leavening agents (such as sodium bicarbonate or potassium bicarbonate),
can promote the release of carbon dioxide over times and the presence of these compounds can tune the pH of the solution and the Maillard reaction.

- The hydrolysis of APs by Faox enzymes, although no clear mechanisms have been demonstrated, could be due to a temporary local unfolding of the protein which allows the glycated site to fit into the catalytic cavity of the enzyme. The interactions between enzyme and Amadori compounds occurs immediately after the carbonyls attachments and they depend on the local steric hindrance. In any case the action of the enzyme resulted in a reduction of the overall extent of MR measured by the key markers of the reaction only when the concentration is relatively high i.e. 1:1000 FAOX: total proteins. This observation points out the importance of the interaction between components that could be verified only when a significant concentration is reached.

- The OMWW secoiridoids are released from the maltodextrin coating. Along with the trapping effect on dicarbonyls, they need to be converted into quinone to form iminoquinone and iminophenol. The direct interaction between quinone and amino group and phenolic rings and dicarbonyls is mandatory. The presence of polyphenols and the *polar paradox* can introduce another important strategy in the control of the MR and lipid oxidation: the location of antioxidant molecules at the interface or inside the lipids droplets can catalyze nucleophilic substitution, dicarbonyls trapping and quinone formation with direct implication for the final extent of the MR. Specifically, non-polar antioxidants are more effective in oil-in-water emulsions since they are retained inside the emulsion droplet where oxidation and the MR is most prevalent.\textsuperscript{292} These systems may offer an intriguing hypothesis where the non-polar phenolic rings can be effective in limiting the glycoxidation effects of reducing sugars.

- The lipidic interface of microemulsions surrounds aliphatic and aromatic amino acids chain influencing the reaction rates and modulating the formation of the APs. The location of amino acids according to the partition coefficient is the key to foresee the extent of the effects. Microemulsions are the closest systems to real food: natural and processed foods consist either partly or wholly as emulsions, or come from emulsified state. They include milk, cream, fruit beverages, infant formula, soups, cake batters, salad dressings, mayonnaise, cream-liqueurs, sauces, deserts, salad cream, ice cream, coffee cream, spreads, butter, and margarine. Despite the huge variety of emulsified foods, there is a considerable lack of knowledge on the Maillard reaction in either model systems and food, with the exception of flavor formation.\textsuperscript{83}
The effects of parameters, such as the dimension of the droplets and the effect of the pH has to be investigated. At pH above 12 the carboxylic group of the amino acids is negatively charged and the partition coefficient of the amino acids increases. This results in the migration of the amino acids at the interface or inside the emulsions with the consequent isolation of carbonyls: the hypothesis is a direct relationship between the pH, location and reaction pathways.

The study of the MRPs formation in emulsions has to consider the interplay between the MR and lipid oxidation, the formation of common intermediates highlighted the possibility of similar reaction pathways that lead to common end-products: i.e. N-substituted hydroxyalkylpyrroles, glyoxal and methylglyoxal follow similar pathways both in the lipid oxidation both in the Maillard pathway. This is a very good reason for the simultaneous evaluation of the extent of lipid oxidation and Maillard reaction in microemulsion based systems.

Multiresponse modeling can provide new insights in the reaction mechanisms. The effects of reducing sugars location upon their degradation and isomerization needs to be developed in relationship to the presence of lipidic phase and to the presence of emulsifiers: by this strategy it will be possible to estimate the formation of the Schiff base and the melanoidins and color formation according to the pH and the thermal treatments.

The location of end-products and brown pigments and the transfer rate of volatile from lipid droplets interface, water and air are all parameters not usually considered in the MR studies. The final results will lead to unravel the reaction mechanisms in emulsion-containing foods.

Future recommendation and forthcoming strategies: b) the chemometric approach

The use of polyphenols and Faox enzymes, as well as the use of encapsulated reactants can be further investigated by following a chemometric approach and response surface methodology (RSM). Firstly, the effects of Faox enzymes in the control of flavor development can be evaluated by several techniques, such as multivariate analysis of variance (MANOVA), principal component analysis (PCA), factor analysis (FA), cluster analysis (CA), and artificial neural networks (ANN), principal component regression (PCR) and partial least-squares regression (PLSR) can combine the
monitoring of several classes of compounds and their relationship to the Fa ox activity. For instance, preliminary PCA exploratory analysis have revealed that the presence of Fa ox is characterized by higher concentration of acetic acid, dicarbonyls and other autoxidation products likely deriving from the degradation of 1-deoxyglucosone. The next step will be the construction of a solid chemometric study able to classify the presence of the enzyme and its relationship to flavor development. Secondly, as mentioned above, the activity of Fa ox should be investigated in relationship to the presence of free amino acids and Amadori compounds. In this frame RSM can clarify the link between dose/response effects promoting the optimization of Fa ox concentration toward protein and free Amadori compounds. Thirdly, the use of the techniques presented in this thesis faces to new analytical instrumentations and to the amount of data that they are able to provide. During the last years, high resolution mass spectrometry with Orbitrap, DART, TOF or FTIR detection, have introduced new insights related to the data representation and data analysis where with one run is possible to obtain multiple information. Despite the organization and visualization of data is a mandatory step, the use of chemometric is the consequence when the activities of hundreds of molecules are measured, as a function of a specific treatment (i.e. Fa ox, polyphenol additions, functional capsules). Several methodologies have been introduced, among them the use of hierarchical clustering and classification can be applied to distinguish the molecular classes influenced by the use of polyphenols. The goal of classification, also known as supervised pattern recognition, is to provide a model that yields the optimal discrimination between several classes in terms of predictive performance. Two case studies have been hypothesized already: A) the presence of polyphenols and the reduction of off-flavor can be monitored by analyzing the consequences on all volatiles formed without focusing on few markers; B) the presence of Fa ox I and II and their de-glycating activity can be evaluated by monitoring the free Amadori compounds present in a specific food matrix.

Promoting the quality of processed foods through the Maillard reaction

Due to the complexity of chemical changes upon thermal treatments or during prolonged storage, rational approaches are required for the promotion of the quality of processed food through the Maillard reaction. Van Boekel and co-workers resumed the aspects that directly impact food processing, where the most important benefits include: food safety, the improvements in the nutritional value; sensory quality, functional health benefits and several economic issues related to economy of scale, seasonality, supply, preparation and convenience. It is not possible to
establish a direct relationship between thermal treatments and food quality; however, generally, there is an optimum after which the increase in the thermal loading corresponds to a decrease of the food quality. Since Louis Camille Maillard monitored the brown color development, food scientists have started facing to the study of the chemical mechanisms beside it, in order to get the browning reaction under control. The ultimate goal is as precise as challenging: the promotion of food quality. It derives that the control of the Maillard reaction reflects complex dynamics in the promotion of desired attributes where a myriad of routes is possible. A multitude of compounds are rearranged and molecules are created. Three aspects concur to determine the relationship between thermal treatment, MR, molecules formed and final quality.294

A) From the fact that “we also eat with our eyes”, the significance of Maillard browning in processed foods and in consumer acceptance is obvious and it relies on the physical modification and on the color formation.

B) The formation of characteristic aromas and flavors obtained upon cooking, baking and roasting is a prerequisite for the acceptance of the foods.

C) On the contrary, of the most obvious negative consequences of the Maillard reaction in food is the loss of nutritive value of proteins involved, with a loss of quality and a possible decrease of food safety.295

In conclusion, the MR goes hand in hand with food quality and beside the array of reactions that governs the final acceptability of the products, the improvements of the MR mitigation strategies represent the easiest way to promote food quality. In Figure 6.6 the several strategies which have been proposed were summarized, among them, four novel tools have been presented in this thesis.
Future works will be devoted to expanding knowledge on the chemical structure of MRPs and their implication in food quality and in biological effects after dietary intake. As examples, the elucidation on melanoidins structures, the reactivity of oligosaccharides, the biological significance of dietary AGEs, the behavior of Maillard precursors in emulsion environment are desirable. The development of innovative technologies that will mitigate harmful compounds while enhancing or without altering the contents of health beneficial compounds will be enabled, thereby maintaining or even improving the nutritional and sensorial properties of processed foods.
Summary

The thesis entitled “New tools in modulating Maillard reaction from model systems to food” aim at answering the following research questions:

- How the control of the MR can be tackled by chemicals, physical and biochemical tools, such as coatings, polyphenols, microemulsion and enzymes?
- Can reactants location modulate the formation of specific key intermediates and the final result of the MR?

MR location and interaction of reactants have been investigated as overall factors supervising the above mentioned targets.

In the first chapter a general introduction on the MR in foods was presented with particular attention to the relationship between MRPs and food quality. In chapter 2 an overview of the control of the MR in foods by using the encapsulation of sodium chloride, ascorbic acid, PUFA and iron was highlighted, beside an introduction of previous mitigation strategies. Encapsulation, widely applied in Food Science and in Food Industry, has been evaluated as a possible tool for the controlled release of reactants over prolonged periods of time or under specific temperatures. The main results were not confined to the preservation of reactants but an additional goal has been achieved as the encapsulation of reactants tuned the final extent of the MR without affecting the sensorial properties of biscuits and other foods. Encapsulation exhibited several potentialities: according to the matrix and to the chemical behavior of the core compounds it was verified that several foods could be tested.

In the third chapter, the use of Faox enzymes was tested in model system consisting in β-lactoglobulin and glucose and in a low lactose milk. Despite Faox was already used for the conversion of Amadori compounds in free amino acids and 1-deoxyglucosone, for the first time the activity towards a complex mixture represented by low lactose milk was reported. Even if the mechanism of action is still unclear, a significative reduction of CML and bound-HMF was reported both in model systems both in low lactose milk after seventeen days at 37°C.

In chapter four the use of a spray dried olive oil mill wastewater powder was tested in lab scale UHT milk and all the steps of the Maillard reaction were investigated. After the characterization of the powder and the quantification of major secoiridoids, it was proven that hydroxytyrosol, tyrosol
and verbascoside were effective in the reduction of dicarbonyls, hydroxycarboxyls, off-flavor and bound Maillard reaction products. A mechanism of action was also hypothesized: the phenolic ring is able to trap the carbonyls and after the oxidation to quinone, primary amino groups and phenols can lead to the formation of iminoquinone and iminophenol.

A preliminary overview on the reaction mechanisms between aromatic amino acids and aliphatic amino acid and glucose in microemulsions was evaluated in chapter five. Data showed that the partition coefficient played a key role by promoting the migration of leucine at the interface of the emulsion droplets, while phenylalanine was mainly located outside the droplets. The formation of the two Amadori compounds (N-(1-deoxy-D-fructos-1-yl)-L-phenylalanine, Fru-Phe and N-(1-deoxy-D-fructos-1-yl)-L-leucine Fru-Leu, respectively) was monitored by high resolution mass spectrometry; the results revealed that even if a sterilization-mimicking thermal treatment the was used (131 °C for 8 min) the formation of Fru-Ile was reduced up to 50 % in presence of microemulsion system, while phenylalanine was scarcely influenced by the lipids droplets and the kinetic profile was substantially similar in control and microemulsions samples.

Despite the four strategies are characterized by methodological differences, they all highlighted a new possible approach. The control of the MR was achieved throughout each stage and the mitigation strategies involve a wide pattern of molecules, without focusing on only one outcome. The tuning of the MR along with the reduction of potentially toxic molecules and the promotion of desired molecules formation is the easiest way to get foods of better quality.
mitigation strategies involve a wide pattern of molecules, without focusing on only one outcome. A new possible approach. The control of the MR was achieved throughout each stage and the kinetic profile was substantially similar in control and microemulsions samples.

Data showed that the kinetic profile was substantially similar in control and microemulsions samples. Despite the four strategies are characterized by methodological differences, they all highlighted a kinetic profile was substantially similar in control and microemulsions samples.
References


References

42. Gokmen, V.; Senyuv, H. Z., Effects of some cations on the formation of acrylamide and furfurals in glucose-asparagine model system. European Food Research and Technology. 2007, 225, 815-820.


44. Morales, F.; Capuano, E.; Fogliano, V., Mitigation strategies to reduce acrylamide formation in fried potato products. Maillard Reaction: Recent Advances in Food and Biomedical Sciences. 2008, 1126, 89-100.


References


68. Soliva-Fortuny, R.; Balasa, A.; Knorr, D.; Martin-Bellos, O., Effects of pulsed electric fields on bioactive compounds in foods: a review. Trends in Food Science & Technology.2009, 20, 544-556.


References


References


201. Sebekova, K.; Somoz, V., Dietary advanced glycation endproducts (AGEs) and their health effects--PRO. *NUTR Food Res.* 2007, 51, 1079-84.

202. Thormalley, P. J., Dietary AGEs and ALEs and risk to human health by their interaction with the receptor for advanced glycation endproducts (RAGE)--an introduction. *NUTR Food Res.* 2007, 51, 1107-10.


References


<table>
<thead>
<tr>
<th>Area</th>
<th>Activity description</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discipline specific activities</td>
<td>11&lt;sup&gt;th&lt;/sup&gt; Symposium International on Maillard Reaction - IMARS and Université de Lorraine (Nancy, France)</td>
<td>09-2012</td>
</tr>
<tr>
<td></td>
<td>17&lt;sup&gt;th&lt;/sup&gt; EuroFoodChem (Istanbul, Turkey)</td>
<td>10-2013</td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; International Conference on Cocoa Coffee and Tea (Naples, Italy)</td>
<td>10-2013</td>
</tr>
<tr>
<td></td>
<td>7&lt;sup&gt;th&lt;/sup&gt; Course “Reaction Kinetics in Food Science“ - Wageningen University (Wageningen, the Netherlands)</td>
<td>10-2012</td>
</tr>
<tr>
<td>General courses</td>
<td>Introduction to MATLAB for Multivariate Data Analysis - University of Copenhagen (Copenhagen, Denmark)</td>
<td>04-2014</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;th&lt;/sup&gt; Italian National Congress on Food Chemistry (Firenze, Italy)</td>
<td>10-2013</td>
</tr>
<tr>
<td></td>
<td>12&lt;sup&gt;th&lt;/sup&gt; Symposium International on Maillard Reaction - IMARS and Tokyo University (Tokyo, Japan)</td>
<td>09-2015</td>
</tr>
<tr>
<td>Optional courses and activities</td>
<td>Non- linear curve fitting as a tool for data evaluation - University of Naples</td>
<td>10-2013</td>
</tr>
<tr>
<td></td>
<td>Advanced course on Mass Spectrometry - University of Naples</td>
<td>10-2012</td>
</tr>
<tr>
<td></td>
<td>PhD course: &quot;How to write a scientific paper“ - University of Naples</td>
<td>06-2013</td>
</tr>
<tr>
<td></td>
<td>Instrumental analysis of flavor compounds - University of Naples</td>
<td>06-2013</td>
</tr>
<tr>
<td></td>
<td>PhD course: &quot;Applied Statistic“ - University of Naples</td>
<td>01-2014</td>
</tr>
<tr>
<td></td>
<td>VLAG PhD week (Venlo, the Netherlands)</td>
<td>04-2014</td>
</tr>
<tr>
<td></td>
<td>Preparing VLAG PhD project proposal</td>
<td>09-2013</td>
</tr>
<tr>
<td></td>
<td>Microfluidics to design encapsulated ingredients - FipDes Master</td>
<td>09-2012</td>
</tr>
<tr>
<td></td>
<td>Omic science in food safety - FipDes Master</td>
<td>09-2012</td>
</tr>
<tr>
<td></td>
<td>Fat emulsions in Mediterranean gastronomy - FipDes Master</td>
<td>11-2012</td>
</tr>
<tr>
<td></td>
<td>Modulation of Maillard reactions in different food preparation : color, texture, aroma - FipDes Master</td>
<td>12-2012</td>
</tr>
</tbody>
</table>

Overview of completed training activities
**List of publications**

Troise Antonio Dario; Fogliano, Vincenzo; Quantitation of acrylamide in foods by High-Resolution Mass Spectrometry - Chapter 25, Acrylamide in foods: analysis, content and potential health effects, 481-494, 2015, Elsevier

Troise, Antonio Dario; Fiore, Alberto; Wiltafsky, Markus; Fogliano, Vincenzo; "Quantification of Nε-(2-Furoylmethyl)-l-lysine (furosine), Nε-(Carboxymethyl)-l-lysine (CML), Nε-(Carboxyethyl)-l-lysine (CEL) and total lysine through stable isotope dilution assay and tandem mass spectrometry", Food Chemistry, 188, 357-364, 2015, Elsevier

Račkauskienė, Ieva; Pukalskas, Audrius; Venskutonis, Petras Rimantas; Fiore, Alberto; Troise, Antonio Dario; Fogliano, Vincenzo; Effects of beetroot (Beta vulgaris) preparations on the Maillard reaction products in milk and meat-protein model systems, Food Research International, 70, 31-39, 2015, Elsevier

Vitaglione, Paola; Troise, Antonio Dario; De Prisco, Anna Chiara; Mauriello, Gianluigi; Gokmen, Vural; Fogliano, Vincenzo; Use of Microencapsulated Ingredients in Bakery Products: Technological and Nutritional Aspects - Chapter 15, Microencapsulation and Microspheres for Food Applications, 303-311, 2015, Elsevier

Troise, Antonio Dario; Fiore, Alberto; Roviello, Giovanni; Monti, Simona Maria; Fogliano, Vincenzo; Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography–high-resolution mass spectrometry, Amino acids, 47, 1, 111-124, 2015, Springer

Colantuono, Antonio; Troise, Antonio Dario; Fiore, Alberto; Fogliano Vincenzo; Phenolic compounds from olive mill wastewater influence N(ε)-carboxymethyl-lysine and N(ε)-carboxyethyl-lysine formation in cookies during baking, IMARS Highlights Commentaries, 9, 6, 5-8, 2014, IMARS
List of publications

Troise, Antonio Dario; Fiore, Alberto; Colantuono, Antonio; Kokkinidou, Smaro; Peterson, Devin G; Fogliano, Vincenzo; Effect of olive mill wastewater phenol compounds on reactive carbonyl species and Maillard reaction end-products in ultrahigh-temperature-treated milk, Journal of agricultural and food chemistry, 62, 41, 10092-10100, 2014, American Chemical Society

Troise, Antonio Dario; Ferracane, Rosalia; Palermo, Mariantonella; Fogliano, Vincenzo; Targeted metabolite profile of food bioactive compounds by Orbitrap high resolution mass spectrometry: the “FancyTiles” approach, Food Research International, 63, 139-146, 2014, Elsevier

Troise, Antonio Dario; Dathan, Nina A; Fiore, Alberto; Roviello, Giovanni; Di Fiore, Anna; Caira, Simonetta; Cuollo, Marina; De Simone, Giuseppina; Fogliano, Vincenzo; Monti, Simona Maria; Faox enzymes inhibited Maillard reaction development during storage both in protein glucose model system and low lactose UHT milk, Amino acids, 46,2,279-288,2014, Springer

de Sereys Liogier, Aliénor; Muller, Sabine; Desic, Sonja; Troise, Antonio Dario; Fogliano, Vincenzo; Acharid, Abdelhaq; Lacotte, Pierre; Birlouez-Aragon, Ines; Potential of the FAST index to characterize infant formula quality - Chapter 28, Handbook of dietary and nutritional aspects of bottle feeding 457 - 475, Wageningen Academy Publisher

Troise, Antonio Dario; Fiore, Alberto; Fogliano, Vincenzo; Quantitation of acrylamide in foods by high-resolution mass spectrometry, Journal of agricultural and food chemistry, 62 ,1, 74-79, 2013, American Chemical Society

Troise, Antonio Dario; Fogliano, Vincenzo; Reactants encapsulation and Maillard reaction, Trends in Food Science & Technology, 33, 1, 63-74, 2013, Elsevier

Fiore, Alberto; Troise, Antonio Dario; Ataç Mogol, Burçe; Roullier, Victor; Gourdon, Anthony; El Mafadi Jian, Samira; Hamzaloğlu, Berat Aýtül; Gökmen, Vural; Fogliano, Vincenzo; Controlling the Maillard reaction by reactant encapsulation: Sodium chloride in cookies, Journal of agricultural and food chemistry, 60, 43, 10808-10814, 2012, American Chemical Society
Acknowledgements

Before starting the writing of this thesis, I had not considered the time I would have needed to dedicate to this paragraph: I was just going to include it among the fastest things to do before printing. I made a terrible mistake!

Now I can firmly say that it is not possible to quantify the time necessary to complete the Acknowledgments of the PhD thesis, just as it is not possible to quantify the time necessary to find the most appropriate word to comment, interpret and present the results of the lab work. This paragraph represents my best attempt at remembering all the people I have met during the last six years since I first started the MSc thesis in the Department of Agriculture in Naples. This is also the best occasion to explain why without “walking for a while” with my colleagues and friends, I probably would have never got these results.

So thank you Vincenzo, because the writing of an article or the writing of a new project have something in common with poems, paintings and sculptures: it is not possible to quantify the time you need to do this. During the last four years, time has become a “dependent variable”: it depends both on the inspiration and on the passion that people put in their scientific work.

Thank you to my friends at the LABS, for giving me the possibility to improve my lab skills and especially for helping me to try and become a scientist. I have to thank each one of them for allowing me to call them “friends” so I will mention them all. Thanks a lot to Lia for her precious help with mass spectrometry; to Alberto Fiore and his special guidance during the first months of my PhD and not only those; to Attilio, Aurora and Giulia, who was the first that introduced me to the world of chromatography; to Daniele “Il Ragioniere” and Antonio, I had the honor to be one of their supervisors, I hope I could give you something back from what the people mentioned above gave me; to Ilario, the perfect companion in this PhD trip since 2012. Special “grazie” to my international friends, Joel, Xianghui and Ezzat for giving me the chance to meet new cultures and for helping me to practice my English. Thanks a lot to Luigi, do not fear, darling, I will continue to disturb you. Many thanks to Paola for being one of my supervisors during the last two years and for her efforts to guide LABS guys.

Thanks to Edo, because his door is always open and I was proud to share with him some ideas; to Claire for accepting my proposal on emulsion and Maillard reaction and for helping me in the
discovery of the importance of emulsion in food chemistry; to Geert, coffee, TSQ and Orbitrap are waiting for us!

Now I have to say thanks to my parents and my loved ones that supported me for the last thirty years. There is not so much space left, but I promise I will personally thank you each one of you. I am going to close this paragraph by thanking two persons, both are part of my family and both represented the most important help also from the scientific point of view. Thanks to my brother Fabrizio, his knowledge of the English language has been the target for my scientific English. There are few words for his patience, two of these are “thank you”. The other person is my wife Serena, with whom I have been walking for more than a while. There are thousands of reasons for which I have to say thank you, but I only chose one: without her passion for science and Chemistry and without her work ethics, my scientific trajectory would not have been possible.
Now I have to say thanks to my parents and my loved ones that supported me for the last thirty years. There is not so much space left, but I promise I will personally thank you each one of you.

I am going to close this paragraph by thanking two persons, both are part of my family and both represented the most important help also from the scientific point of view. Thanks to my brother Fabrizio, his knowledge of the English language has been the target for my scientific English. There are few words for his patience, two of these are “thank you”.

The other person is my wife Serena, with whom I have been walking for more than a while. There are thousands of reasons for which I have to say thank you, but I only chose one: without her passion for Science and Chemistry and without her work ethics, my scientific trajectory would not have been possible.