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Analytical strategies for the monitoring of food quality through the Maillard reaction

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General abstract

The Maillard reaction (MR) supervises the final quality of foods and the study of the underneath reaction mechanisms provides a detailed overview of the thermal impact. The monitoring of Maillard reaction products formation (MRPs) is one of the most challenging procedures in Analytical Chemistry since their detection is highly influenced by the polarity of the compounds, the high reactivity, the complexity of the matrix, and the continuous changes in the reaction mixture. The accurate setup of the analytical methods for the MRPs quantification defines several opportunities for the monitoring of food quality. The MR can be divided into three stages: activation stage, intermediate stage and advanced stage; each of them contributes to the final quality of foods. The analysis of free amino acids and free Amadori compounds by high resolution mass spectrometry provides useful information on the initial stages and the final direction of the reaction can be depicted by monitoring the ratio between free Amadori compounds and free amino acids. Beside the monitoring of the first stages of the MR, the analysis of bound MRPs, furosine, CML, CEL and total lysine by stable isotope dilution assay tandem mass spectrometry offers a snapshot of the glycation on proteins and the loss in the nutritional values of foods. According to the advanced staged, the quantification of acrylamide by high resolution mass spectrometry highlights two novel insights: on one hand the use of some contaminants for the continuous recalibration of the instrument as a tool to reduce the mass error up to 1 ppm also with small amides; on the other hand the possibility to rule out the solid phase extraction for acrylamide detection. Finally, the “FancyTiles” approach reveals the possibility to combine the signals of different MRPs in order to obtain a colorimetric scale typical for a defined process and thermal treatment. These preliminary findings highlights the possibility to create a direct link between MR, chemometric and food quality.

Chapter 1

Introduction

1.1 Quality in Food Science

The American Society for Quality defines quality as “a subjective term for which each person has its own definition based on its experience. Two interrelated aspects concur to the definition of quality: (a) the ability of products or services to satisfy stated or implied needs; (b) a product or service free of deficiencies. In other words, quality is the direct consequence of: ‘fitness for use’ and ‘conformance to requirements’”[1]. These two statements are the background for the definition of the concept of Food Quality. From the point of view of the consumers, expectations are based on manufacturing and processing standards and the main targets are focused on the knowledge of what ingredients are inside, in particular when specific dietary and nutritional requirements are requested. Quality expectations may include external factors such as appearance (size, shape, color, gloss, and consistency), sensory properties (texture, taste, flavors and aroma), nutritive values (chemical constituents), mechanical properties, functional properties, the absence of defects and last but not the least factors such as federal and internal grade standards (chemical, physical, microbial). It derives that the quality of foods is not a single, well-defined attribute but it involves many properties and characteristics. Finally, quality can be simply defined as a human construct for a product comprising many properties and characteristics desired by consumers [2].

On the contrary, beside the consumers point of view, the main targets for food scientists are related to the methods for monitoring food quality, the nutritional quality, shelf life testing and validation; in this respect, environmental factors affecting food quality, the impact of present and proposed regulation and statistical interpretation play a key role [3]. On this basis, incorporating and promoting quality into products and process design is one of the most intriguing challenges for both food manufacturers and food scientists. Products and process designs need to be flexible and valid for several reasons since food quality and safety are of paramount importance for consumers, retailers as well as regulators engaged in enacting food laws.

This scenario implies two aspects. On one hand reaching quality by trials and errors is an expensive way that can be overcome by a more systematic way, such as the use of modeling; in fact, design process can be simulated in virtual labs using quantitative and mathematical models.[4] On the other hands the definition of the reactions that mainly contribute to the final quality of foods can be a powerful tool to verify if a product fits to consumers expectations or to proposed regulations.

The quality of foods changes over time and as a consequence the quality indicators change accordingly. The most important changes involved quality issues are [5]:

- Chemical reactions, mainly due to either oxidation (i.e. lipid oxidation or ascorbic acid oxidation) or Maillard reactions and caramelization.
- Microbial reactions: microorganisms can grow in foods; in the case of fermentation this is desired, otherwise microbial growth will lead to spoilage and, in the case of pathogens, to unsafe food.
- Biochemical reactions: many foods contain endogenous enzymes that can potentially catalyze reactions leading to quality loss (enzymatic browning, lipolysis, proteolysis, and more). In the case of fermentation, enzymes can be exploited to improve quality.
- Physical reactions: many foods are heterogeneous and contain particles. These particles are unstable, in principle at least, and phenomena such as coalescence, aggregation, and sedimentation lead usually to quality loss. Also, changes in texture can be considered as physical reactions, though the underlying mechanism may be of a chemical nature.

In this respect the Maillard reaction becomes the turning point for the creation of a direct relationship between quality and color, taste, aroma, texture, nutritive losses and formation of toxicologically suspect compounds. Moreover, all the biological and chemical changes listed above are tightly connected to the Maillard reaction revealing that this reaction occupies a crucial role in Food Chemistry and Technology. In particular, lipid oxidation and Maillard reaction share common intermediates [6], the presence of microorganisms can modify the pH and influence the formation of certain products related to the Maillard pathways; the presence of emulsions or aggregates can interfere with the reaction rates [7, 8]; some enzymes (i.e. fructosamine oxidase and asparaginase) are valuable tools for the control of the reaction, hence to improve the final quality of processed foods [9, 10].

1.2 The Maillard reaction behind Food Quality

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 [11]. 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 [12]. 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 upon the Heyns rearrangement to the N-substituted D-glucosylamine, an 1-amino-2-deoxy-1-aldhose (HP) is formed [13]. 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-dicarbonyls, 2,3-enolisation via 1-deoxy-2,3-dicarbonyls, 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:

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 [14].

These three 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 [15]. 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. In this frame, a systematic classification is complicate, but a tentative overview of MR volatiles has been proposed by Nursten [15]:

- "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, trithiolanes, dithianes, trithianes, furanthiols.

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, Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL) and lysino-alanine. Since lysine is an essential amino acid and its ε-amino group has special significance, several analytical efforts have been addressed to its quantification. From the chemical point of view, the reaction with glucose gives N-ε- deoxyfructosyl-lysine (fructose-lysine) 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 [16]. 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 [17]. 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 [18, 19]. HMF is a furanic compound that can be formed both from MR both from caramelization and autoxidation of sugars, via 3-deoxyosone formation [20].

1.3 Analytical strategies for the detection of MRPs: a case study

The MR leads to the formation of hundreds of molecules that can be tentatively grouped into two broad categories: desired molecules and undesired molecules, even if a clear distinction is not possible as a consequence of the bewildering array of reactions that contributes to the formation of a defined end-products. The detection and accurate quantification of MRPs is a major challenge since several pathways can occur beside the changes in the matrix, the variation of the pH, the increase

of the temperature, the diffusion of reactants, the coefficients of partition, the presence of lipids, emulsions and micelles. In this complex scenario, the concentration of precursors, intermediates and end-products changes according to the reaction conditions; the high variability of the matrix determines several drawbacks that render the extraction, detection and quantitation of MRPs particularly complex. In the frame of "fast analysis, reliable results," the analytical strategies related to the monitoring of strategic intermediates (i.e. the Amadori compounds), of heat-induced toxicants, such as some of the MRPs (i.e. acrylamide, HMF, CML and CEL) or of the loss of essential amino acids (i.e. lysine) still represents one of the hottest topic of Food Chemistry with several implications in Food Toxicology and Food Engineering and Food Technology. In this respect, the analysis of acrylamide is one of the most significant examples. A brief overview of the analytical improvements is here presented, while the method of choice for its detection by high resolution mass spectrometry will be described in chapter 4.

Since the first report highlighted the presence of acrylamide at high concentration in French fries, cereals based foods, and coffee, the study of aspects linked to its mechanism of formation, chemical kinetics, analytical detection and quantification have become one of the most stressed area in Maillard chemistry. A huge variety of papers dealt with the chemical and analytical insights of acrylamide determination and as a consequence in the past decade several methods for quantitative determination have been reviewed [21, 22]. Acrylamide analysis has three critical steps: sample preparation, including extraction, spiking with labeled internal standard with or without derivatization and cleanup of the samples, chromatographic separation and compounds detection. With regard to the first step several techniques have been developed providing satisfactory results: cation-exchange cartridges, hydrophilic-lipophilic balanced cartridges, and reversed phase C18 cartridges [23].

The chromatographic separations should take into consideration, just like other small molecules with an amide group or an amino group such as glycine, several drawbacks; among them the high polarity as well as the poor retention, matrix effect, and solvent interferences, such as acetonitrile that in the case of mass spectrometry detection without derivatization can easily suppress the current ion associated to the signals [24]. Several efforts can be undertaken in order to rule out these disadvantages. First, the derivatization procedures with mercaptobenzoic acid or through the use of the "bromination" method help in increasing the selectivity in acrylamide determination, in particular with single stage LC mass spectrometers [25]. Second, for the detection step the analytical techniques of choice are: tandem mass spectrometry (MS/MS) acquisition performed by

selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), choosing the characteristic fragmentation pathway [26]; Fourier transform infrared (FT-IR) analysis [27], or time of- flight mass spectrometry (TOF/MS) [28] and fluorescence method [29]. Despite the outstanding results obtained for the quantification of acrylamide in food matrices, there are still several strategies that can offer some advantages to the analytical aspects reviewed up to now. In particular, a promising approach is the use of direct analysis real time (DART) and Orbitrap analyzer, whose specificity and high mass accuracy can be further improved by the use of the internal recalibrating mass. The presence of contaminants in mass spectrometry is a negative outcome and in most cases the effect of the interferences has to be reduced or avoided. By using HRMS, the presence of contaminants with *m/z* near the one of the acrylamide can offer the unique chance to continuously recalibrate the instrument increasing the accuracy and as a consequence reducing the mass tolerance up to 2 ppm. This procedure was described by the group of Matthias Mann for complex peptide mixtures. They highlighted the benefits due to the introduction of an omnipresent contaminant from ambient air as an internal standard which they accumulate together with ions of interest before injecting this mixture into the Orbitrap for the measurement. As described for acrylamide, absolute mass deviations of less than 1 ppm were achieved [30, 31].

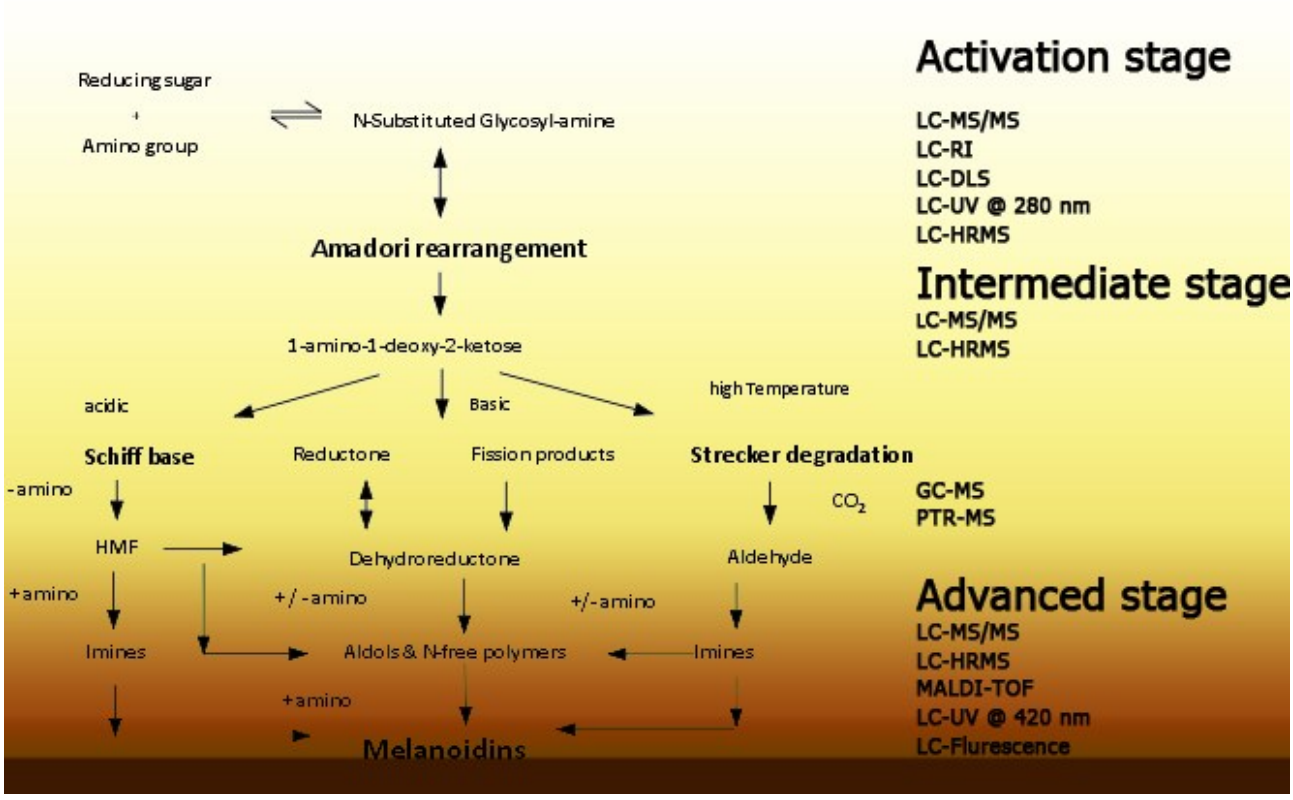

Figure 1.1: Analytical strategies and Hodge schema with color development .

Similarly to acrylamide detection, the same evolution of the analytical strategies can be described for other MRPs. In particular in **Figure 1.1** the Hodge's schema is combined with different approaches for the detection of the class of molecules according to the stages of the reaction [11]. As expected, while for the initial or activation stage the use of liquid chromatography, mass spectrometry and UV detection is the technique of choice in the intermediate and in advances stage the optimal detection for volatiles is achieved by using PTR-MS or GC-MS. The late stage of the MR with the formation of colored compounds can be related to the use of UV detection along with mass spectrometry.

Chapter 2

Simultaneous quantification of amino acids and Amadori products in foods through ion pairing liquid chromatography High Resolution Mass Spectrometry

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Abstract

The formation of the Amadori products (APs) is the first key step of Maillard reaction. Only few papers have dealt with simultaneous quantitation of amino acids and corresponding APs (1-amino-1-deoxy-2-ketose). APs chromatographic separation is affected by several drawbacks mainly related to their poor retention in conventional reversed phase separation. In this paper, a method for the simultaneous quantification of amino acids and their respective APs was developed combining high resolution mass spectrometry with ion pairing liquid chromatography. The limit of detection were 0.1 ng/mL for tryptophan, valine and arginine, while the limit of quantification ranged from 2 and 5 ng/mL according to the specific sensitivity of each analyte. The RSD % was lower than 10% and the coefficient of correlation was higher than 0.99 for each calibration curve. The method was applied to milk, milk based products, raw and processed tomato. Among the analyzed products, the most abundant amino acid was glutamic acid (16646.89 ± 1385.40 μg/g) and the most abundant AP was fructosyl-arginine in tomato puree (774.82 ± 10.01 μg/g). The easiness of sample preparation coupled to the analytical performances of the proposed method introduced the possibility to use the pattern of free amino acids and corresponding APs in the evaluation of the quality of raw food as well as the extent of thermal treatments in different food products.

Keywords: Maillard reaction, amino acids, Amadori products, high resolution mass spectrometry

2.1 Introduction

The central hub of the Maillard Reaction (MR) is represented by the reaction between reducing sugars, such as D-glucose, and amino acids and proteins: this step is the basement from which the Maillard cascade starts off [11]. Since the first articles by Nordin and Amadori highlighted the reaction between carbonyls and amines [32, 33], many papers have dealt with this complex reaction in order to evaluate the key steps of the beginning of the MR. The initial phase is characterized by the formation of a cyclized Schiff base, a glycosylamine that via immonium ion can generate alternatively the α and β anomers. The acidic condition promotes the rearrangement of the N-glycosides where the isomerization reaction leads to the synthesis of the more stable 1-amino-1-deoxy-2-ketose; this process is known as Amadori rearrangement if the reducing sugar is an aldose or Heyns rearrangement if the reducing sugar is a ketose. The final result of this reaction is the formation of the Amadori rearrangement products (APs) or Heyns rearrangement products (HPs, 1-amino-2-deoxy-1-aldose) as it is shown in **Figure 2.1** [12, 13]. The extent of APs and HPs concentration depends on the chemical reaction leading to their formation which include 1,2- and 2,3-enolisation, β-elimination, the migration of the carbonyl group and retroaldol reactions [34]. The APs formation was mainly investigated under the mild conditions typical of long term stored food, the high temperature/short time of the UHT treatment but also under the physiological condition of living organisms [27, 35-39].

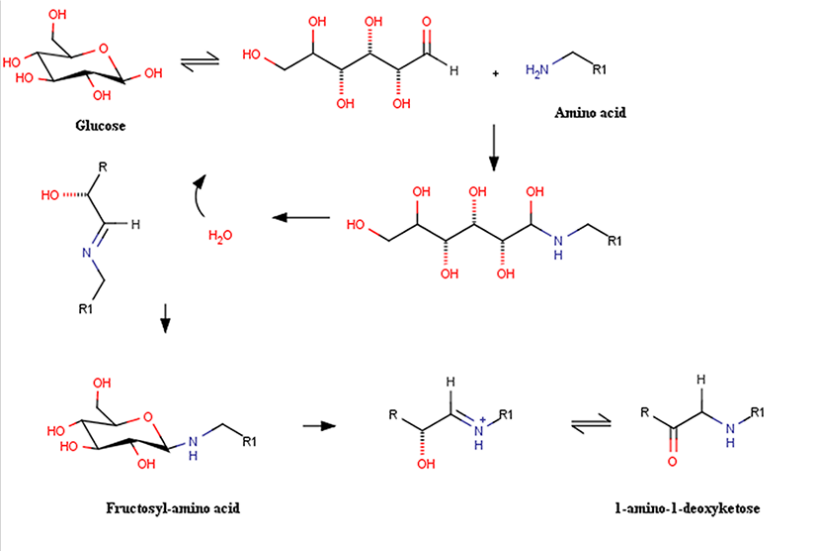


Figure 2.1: Amadori product formation, adapted from Yaylayan and Huyghuesdespointes [13].

A complex deal for food chemistry is the simultaneous detection of free amino acids and their respective APs since it faces many difficulties such as the high polarity, the chemical and chromatographic behavior, the derivatization procedures required for their detection [13, 40-42]. A number of papers reported the detection and quantification of free amino acids in foods and in biological fluids [43, 44]. Focusing only on HPLC based procedures several fully validated methods involve many different techniques: post-column and pre-column derivatization with UV or fluorescence detector [45-48]; mass spectrometry with single or triple quadrupole [49-52], high resolution mass spectrometry with time-of-flight (TOF), Fourier-transform ion cyclotron resonance (FTICR) or high resolution mass spectrometry (HRMS) analyzer [53-55]. This last technique offers some advantages overcoming the most common problems related to the constrains mentioned above. The full spectra acquisition mode and tandem mass spectrometry allow a simplified extraction and a short chromatographic approach while the high resolution enables unambiguous identification of amino acids matching the theoretical *m/z* with experimental *m/z* up to fifth decimal digits [55].

HRMS-based methods can also be applied to the quantification of APs which is still one of the most desired goals of those investigating the MR. The chemical features along with the lack of commercial source of APs standards represent the first disadvantages for the building of a fully validated method. Moreover, the chemical or enzymatic hydrolysis necessary to quantify the APs bound to the proteins introduce high uncertainty and variability. Some of the strategies used for quantification of APs, mainly of Nε-(1-Deoxy-D-fructos-1-yl)-L-lysine or glycated proteins, are summarized **Table 2.1**.

Traditionally, the APs was indirectly quantified through furosine analysis [56, 57], even if the acidic hydrolysis, the MR extent and the similarity between lactosylamine and Nε-(1-Deoxy-D-fructos-1-yl)-L-lysine negatively influence the conversion of furosine into APs [58]. Several strategies have been evaluated for the detection of APs in food including a large use of mass spectrometry detection [59-63] and HPLC diode array detection via furoylmethyl derivatives formation [64, 65]. The analysis of the typical fragmentation pathway and the neutral loss scan of APs by tandem mass spectrometry along with gas or liquid chromatography are the key procedures which have been extensively applied [12, 66-68].

In this paper we used HRMS combined with ion pairing liquid chromatography to develop a method for the quantification of free amino acids and corresponding APs using a fast sample

preparation and without any derivatization. The simplified approach with a linear chromatographic gradient and the full spectra scanning and acquisition mode allowed a rapid and sensitive analysis

Table 2.1: Overview of Amadori products detection methods previously reported

Marker	Technique	Detection
β-Lactoglobulin	Neutral loss scan	LC/ESI-MS/MS
Glycated tetrapeptide, Fru-Lys	Enzymatic hydrolysis and LC	LC/MS
Fru-Gly	GC and Pyrolysis/GC	GC/MS
Fru-Glu, Fru-Pro	High-performance ion exchange chromatography	ESI-MS/MS and ESI-MS
Fructosamino-, ribulosamino, and glucosamino- modified peptides	Positive ion-mode	nanoESI-QqTOF- and MALDI-TOF/TOF-MS
Fru-His and Fru-Arg	Gas/Liquid Chromatography; Trimethylsilyl Derivatives	GLC-MS/MS
	Neutral loss scan	RP-LC/ESI/MS/MS Q-ToF
Fru-Lys	SPE/ Ion pairing liquid chromatography	LC/ESI-MS/MS
Lactulosyl-L-lysine		MALDI-TOF-MS
Fru-Gly; Fru-Asn	LC-ESI-MS/MS	MS/MS; MRM mode
Fru-Phe, Fru-Met, Fru-His, Fru-Ile, Fru-Leu, Fru-Val, Fru-Tyr	Stable isotope dilution assay; positive ion mode	LC/MS/MS

2.2 Material and methods

2.2.1 Chemicals

Acetonitrile and water for LC/HRMS analysis along with methanol and acetic acid for fructosyl-L-amino acids synthesis were obtained from Merck (Darmstadt, Germany). The ion pairing agent perfluoropentanoic acid (NFPA), all the standard amino acids, D-glucose, sodium pyrosulfite, dimethylformamide, morpholine, sodium hydroxide and Nα-Fmoc-protected lysine were purchased from Sigma-Aldrich (Saint-Louis, MO). The calibration solution (see "liquid chromatography/high resolution mass spectrometry" section) was obtained from Thermo Fisher Scientific (Bremen, Germany).

2.2.2 Fructosyl-L-amino acids (Amadori Products, APs) synthesis

Fructosyl-L-amino acids (Nε-(1-Deoxy-D-fructos-1-yl)-L-amino acids) were prepared refluxing in methanol a mixture of amino acid and an excess of anhydrous D-glucose following the course of the

reaction by TLC accordingly with the synthetic protocols previously described in literature for the various derivatives. Specifically, fructosyl-L-histidine and fructosyl-L-asparagine were synthesized according to the procedure of Mossine and Mawhinney [69], using a 3.3:1 glucose/amino acid molar ratio in the presence of sodium pyrosulfite and acetic acid, whereas fructosyl-L-phenylalanine was obtained as described by Glinsky et al. by using a 4:1 glucose/phenylalanine ratio [70]. The preparation of fructosyl-L-aspartic acid was performed in analogy to the synthetic route presented by Abrams et al. by refluxing the mixture of L-aspartic acid and D-glucose in the presence of one mole of sodium hydroxide per mole of amino acid [71]. In case of fructosyl-L-lysine, the synthesis of which was accomplished by the two-step procedure described by Vinale et al. (1999) starting from a Nα-Fmoc-protected derivative, a further step for the removal of the Fmoc moiety was performed by treating the Nα-Fmoc-protected fructosyl-L-lysine with morpholine (8 equivalents) in a 9:1 dimethylformamide/methanol mixture [72]. The results of the structural analysis are in good agreement with previously reported criteria of purity and structural confirmation for all the fructosyl-amino acids. Each amino acid was named using the three letter code while the respective Nε-(1-Deoxy-D-fructos-1-yl)-L-amino acids (AP) were identified using the three letter code along with the notation “Fru-”.

2.2.3Food samples

Amino acids and APs were monitored in different food products: UHT milk, low lactose milk, milk based caramel powder and raw tomatoes (three varieties: “Locale Chieti”, “Locale Lazio” and “Stella Pisa”, named T1, T2, T3, respectively). Furthermore the method was validated in three different canned tomatoes samples: Cherry tomatoes (CT), yellow Cherry tomatoes (variety: “Datterini”, YDT), and in a tomato paste (TP) . All the samples were purchased in a local market and a simplified sample preparation procedure was followed. Tomato samples were ground in a knife mill Grindomix 200 (Retsch, Haan, Germany) and 100 mg were mixed with 0.3 mL of deionized water; 0.1 mL of UHT milk and low lactose milk were diluted three times in water, while 100 mg of milk based caramel powder were mixed with 0.9 mL of water . Samples were centrifuged (14800 rpm, 20 min, 4°C), then the supernatants were filtered using regenerated cellulose filters (RC 0.45 μm, Phenomenex, Torrance, CA) and analyzed.

2.2.4 Liquid Chromatography High Resolution Mass Spectrometry (LC/HRMS)

For the chromatographic separation of amino acids and their respective APs, the mobile phases consisted of 5 mM perfluoropentanoic acid (NFPA) in water (solvent A) and 5 mM NFPA in acetonitrile (solvent B). The following linear gradient of solvent B (min/%B): (0/2), (2/2), (5/50), (7/50), (9/2), (12/2), (15/2) was used. The flow rate was set to 200 µL/min and the injection volume was 5 µL. Chromatographic separation of amino acids and APs was achieved through a thermostated (30 °C) Kinetex 2.6 µm (100 x 2.1 mm) core shell C-18 column (Phenomenex, Torrance, CA). The Accela 1250 UPLC system (Thermo Fisher Scientific, Bremen, Germany) was directly interfaced to an Exactive Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and analytes were detected through a heated electrospray interface (HESI) operating in the positive mode and scanning the ions in the *m/z* range of 60–500. The resolving power was set to 50000 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 x 10^6 ions); maximum injection time was 50 ms. The interface parameters were as follows: spray voltage 3.8 kV, capillary voltage 10 V, skimmer voltage 15 V, capillary temperature 275 °C, heater temperature 200 °C, sheath gas flow 30 and auxiliary gas flow 3 arbitrary units. 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.

2.2.5 Method performances

A stock solution of each amino acid listed in **Table 2.2** was prepared dissolving 10 mg of each standard in 1 mL of a mixture of water/acetonitrile/acetic acid (90.00/9.99/0.01). Each solution was diluted and stored at -20°C until usage. Before starting the analysis two different mixtures one with amino acids and another with APs were prepared and HRMS method was tuned by infusion of these two mixtures first directly in the ion source, then in the chromatographic stream. Two sets of calibration curves for the 20 amino acids and for the 5 APs were built in the range 5 - 5000 ng/mL according to the limit of detection (LOD) and to the limit of quantitation (LOQ) [73]. Three replicates of 0.5 ng/mL solutions were injected into the LC/HRMS system to verify the lowest concentration for which the signal to noise ratio was higher than three. The r^2 value was calculated plotting the area counts against the injected concentration and it was always higher than 0.99 in the

above mentioned range. Each point of the calibration curves was injected three times in the same day (intraday assay for the repeatability) and three times in three different days (interday assay for the reproducibility): the accuracy was reported as the discrepancies between six calibration curves each of them performed intraday and interday. The slope (or the sensitivity of the method) among the six curves was performed and compared to each point of each calibration curve. The results were expressed as relative standard deviation RSD (%). The concentration of non-synthesized APs was calculated according to the chemical class of each amino acid as listed in **Table 2.3**: the calibration curve of Fru-Lys was used for Fru-Arg and Fru-Pro; Fru-Phe was used for all hydrophobic and aromatic APs and, Fru-Gln was calculate by Fru-Asn calibration curve and Fru-Glu by Fru-Asp. Fru-Cys, Fru-Met, Fru-Thr and Fru-Ser and Fru-Gly were calculated using the calibration curve of Fru-Asn. The recovery test was performed spiking a mixture of tomato juice with a known amount of amino acids and APs (final concentration 1µg/mL) and taking into account the overestimation due to amino acids and APs already present in the sample. The recovery was calculated plotting the area of the current associated to each m/z of each amino acid and Amadori products in the tomato matrix (with and without the standard) towards the concentration of the standard according to the following formula:

$$R = \left(\frac{C_a}{C_s}\right) \times 100 = \frac{(C_o - C_b)}{C_s} \times 100$$

Where R is the recovery; C_a is the concentration of the spiked analyte in the samples, C_s is the concentration of the spiked solution; C_o is the observed concentration of the spiked matrix and C_b is the basal concentration of each compound without the spiked solution.

The tolerance range for mass accuracy of the amino acids and APs (i.e. the experimental mass of each analyte had to fall within the maximum permitted tolerance) was fixed at 5 ppm [74]. Each sample was injected four times and the results were reported as µg/g FW (fresh-weight) for tomatoes and µg/mL of samples for milk liquid products according to the matrix. Data were recorded and analyzed using Xcalibur software version 2.1 (Thermo Fisher Scientific, Bremen Germany), while the FancyTyle schemas were built using XLStat 3D-Pro statistical software (Addinsoft, New York, NY)

Table 2.2: High Resolution Mass Spectrometry (HRMS) performances of amino acids and Amadori products. Rt (retention time, min); error (ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass. This ratio was multiplied per one million in order to obtain the ppm. All the analytes were detected in positive ionization mode ($[M+H]^+$). AA: amino acids; APs (Amadori products)

Type	AA	Rt	Exact mass $[M+H]^+$	Experimental mass $[M+H]^+$	Error (ppm)	APs	Rt (min)	Exact mass $[M+H]^+$	Experimental mass $[M+H]^+$	Error (ppm)
Aliphatic	**Ala**	2.41	90.05496	90.05529	-3.66	**Fru-Ala**	1.65	252.10778	252.1077	0.33
	Val	5.31	118.08626	118.08645	-1.61	**Fru-Val**	2.79	280.13908	280.1389	0.65
	Leu	6.47	132.10191	132.1019	0.08	**Fru-Leu**	5.35	294.15473	294.15469	0.15
	Ile	6.35	132.10191	132.1019	0.08	**Fru-Ile**	5.35	294.15473	294.15469	0.15
Aromatic	**Phe**	6.65	166.08626	166.08641	-0.90	**Fru-Phe**	6.12	328.13908	328.13876	0.99
	Trp	6.90	205.09715	205.09738	-1.12	**Fru-Trp**	6.79	367.14997	367.14924	2.00
	Tyr	5.80	182.08117	182.08125	-0.44	**Fru-Tyr**	2.92	344.13399	344.1338	0.56
Polar	**Cys**	2.00	122.02703	122.02751	-3.93	**Fru-Cys**	1.48	284.07985	284.07955	1.07
Neutral	**Met**	5.54	150.05833	150.0584	-0.47	**Fru-Met**	2.96	312.11115	312.11219	-3.32
	Ser	1.89	106.04987	106.05009	-2.07	**Fru-Ser**	1.45	268.10269	268.10344	-2.79
	Thr	2.06	120.06552	120.06564	-1.00	**Fru-Thr**	1.46	282.11834	282.11838	-0.13
	Asn	1.83	133.06077	133.06091	-1.05	**Fru-Asn**	1.48	295.11359	295.11350	0.32
	Gln	1.92	147.07642	147.07651	-0.61	**Fru-Gln**	2.78	309.12924	309.12969	-1.45
Acidic	**Asp**	1.77	134.04478	134.04491	-0.97	**Fru-Asp**	1.37	296.09760	296.09761	-0.02
	Glu	2.15	148.06043	148.06051	-0.54	**Fru-Glu**	1.69	310.11325	310.11377	-1.67
Basic	**Arg**	6.59	175.11895	175.11879	0.91	**Fru-Arg**	5.43	337.17177	337.17172	0.16
	His	6.08	156.07675	156.07686	-0.70	**Fru-His**	4.80	318.12957	318.12973	-0.49
	Lys	6.41	147.11280	147.11266	0.95	**Fru-Lys**	5.35	309.16562	309.16562	0.01
Unique	**Pro**	2.11	116.07061	116.07086	-2.15	**Fru-Pro**	1.54	278.12343	278.12341	0.08
	Gly	2.09	76.03930	76.03964	-4.47	**Fru-Gly**	1.57	238.09212	238.09186	1.11

Table 2.3: Amino acids and Amadori Products analytical performances, LOD (limit of detection, ng/ml), LOQ (limit of quantification, ng/ml); r^2 mean of the coefficient of correlation of the six calibration curves performed; RSD (%), relative standard deviation among the calibration curves. For the not synthesized APs the reference APs used for the calibration curve was inserted. 1: quantified using Fru-Phe as reference curve; 2: quantified using Fru-Asn as reference curve; 3: quantified using Fru-Asp as reference curve; 4: quantified using Fru-Lys as reference curve.

Amino acids	LOD	LOQ	r^2	RSD (%)	Recovery	APs	LOD	LOQ	r^2	RSD (%)	Recovery
Ala	0.5	2	0.990	7.01	105	**Fru-Phe**	1	5	0.998	7.49	90
Val	0.1	2	0.986	5.51	89	**Fru-Asn**	0.5	5	0.989	8.30	88
Leu	0.5	2	0.987	4.98	90	**Fru-Asp**	1	5	0.985	8.31	95
Ile	0.5	2	0.987	4.98	90	**Fru-His**	2.5	5	0.991	4.32	92
Phe	0.5	2	0.992	7.25	101	**Fru-Lys**	1	5	0.990	7.31	95
Trp	0.1	2	0.994	5.35	95	**[1]Fru-Ala**	1	5	0.998	7.49	90
Tyr	0.5	2	0.990	4.21	91	**[1]Fru-Val**	1	5	0.998	7.49	90
Cys	1.0	5	0.988	7.89	85	**[1]Fru-Leu**	1	5	0.998	7.49	90
Met	1.0	5	0.991	7.55	101	**[1]Fru-Ile**	1	5	0.998	7.49	90
Ser	0.5	2	0.989	6.88	98	**[1]Fru-Trp**	1	5	0.998	7.49	90
Thr	0.5	2	0.993	4.01	92	**[1]Fru-Tyr**	1	5	0.998	7.49	90
Asn	0.5	2	0.988	5.53	91	**[2]Fru-Cys**	0.5	5	0.989	8.30	88
Gln	0.5	2	0.986	3.30	92	**[2]Fru-Met**	0.5	5	0.989	8.30	88
Asp	0.5	2	0.991	8.01	95	**[2]Fru-Ser**	0.5	5	0.989	8.30	88
Glu	0.5	2	0.997	6.05	95	**[2]Fru-Thr**	0.5	5	0.989	8.30	88
Arg	0.1	2	0.987	6.09	88	**[2]Fru-Gln**	0.5	5	0.989	8.30	88
His	0.5	2	0.993	5.55	89	**[3]Fru-Glu**	1	5	0.985	8.31	95
Lys	0.5	2	0.991	7.21	105	**[4]Fru-Arg**	1	5	0.990	7.31	95
Pro	1.0	2	0.993	7.01	100	**[4]Fru-Pro**	1	5	0.990	7.31	95
Gly	0.1	2	0.991	6.55	101	**[2]Fru-Gly**	0.5	5	0.989	8.30	88

2.3 Results and discussion

2.3.1 LC/HRMS analysis

Pure APs used in the present study were obtained following well-established synthetic protocols previously described in literature for the various compounds. All the above mentioned parameters were checked to ensure the highest signal and the lowest mass error. Two different solutions of standard amino acids and APs were infused first directly into the ion source then into the chromatographic flow in order to tune the optimal detection conditions and to investigate the interferences due to the solvent and to the ion pairing agent. The mass spectrometry conditions were

optimized to avoid any in source fragmentation. Taking into account the specificity of each analyte, it was decided to use a low potential, of 15 V and 10 V for capillary voltage and skimmer voltage, respectively and slow flow of auxiliary gas. These conditions avoided the fragmentation of the molecules filling the C-trap (number of ions injected for each scan: 1 x 10^6) with the parental ions without the formation of daughter ions ensuring the highest abundance of each signal [61]. The presence of a recalibrating agent such as diisooctyl phthalate favored the reduction of the discrepancies between the theoretical mass and the experimental mass. The mass error was always in the range ± 5 ppm; the highest was that of glycine, with a mass error of -4.47 ppm likely due to its low *m/z.*, switching the recalibrating agent to low *m/z* it should be possible to reduce the error also for glycine [75]. The specificity and the chemical features of each analyte was one of the crucial point of this study: the above described conditions proved to be optimal for the simultaneous detection of amino acids and APs. In **Figure 2.2 and 2.3** the typical chromatographic profiles of amino acids and APs were reported. The pair ions with an opposite charge improved the retention of the target molecules and the separation of free amino acids from the APs. The retention pattern followed the typical reversed phase behavior: polar amino acids eluted first, while the hydrophobic and the side chain charged amino acids eluted later ones. Interestingly, all APs eluted before the respective amino acids, according to the increase of the polar solubility due to the ketose or aldose residues [13].

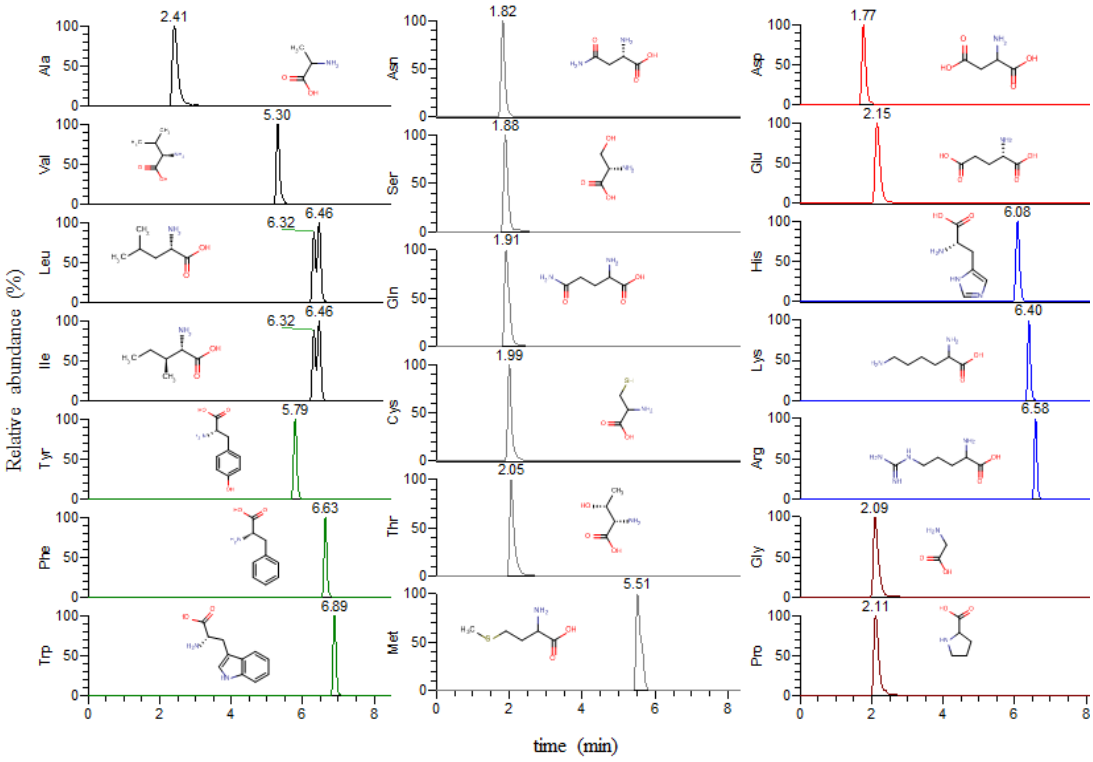


Figure 2.2: Chromatographic profile of amino acids, black (hydrophobic), green (aromatic), gray (polar neutral), red (acidic), blue (basic), brown (unique).

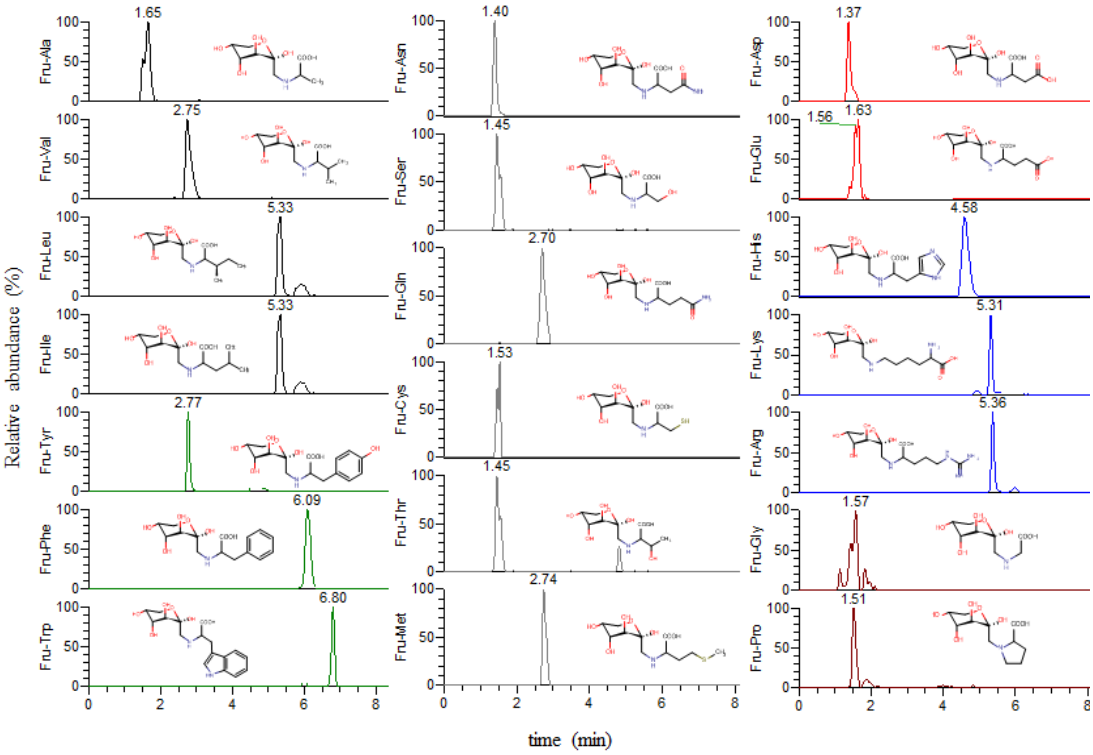


Figure 2.3: Chromatographic profile of Amadori products, black (hydrophobic), green (aromatic), gray (polar neutral), red (acidic), blue (basic), brown (unique).

2.3.2 Method development and performances

Method performance was tested against the following quality parameters: mass accuracy, retention time, carryover, linearity of the calibration, coefficient of correlation (r^2), limit of detection (LOD), limit of quantification (LOQ) repeatability, reproducibility and recovery. The results of the analytical performances are reported in **Table 2.3**. The first point was to check the absence of any contaminant with the same exact mass of the target compounds by injecting several times a solution of water: acetonitrile 70:30. LOD is the lowest analyte concentration to be reliably distinguished from the noise and at which detection is feasible, while LOQ is the lowest concentration at which the analyte can be reliably detected and defined goals for bias and imprecision are acceptable. In other words, in amino acids detection LOQ should be at least five times LOD [73]. After ten replicates the solution 0.1 ng/mL for all the amino acids and for the synthesized APs resulted in no signal, while the linearity was achieved in the range 5-5000 ng/mL and 5 - 2000 ng/mL for amino acids and APs, respectively. The reproducibility and repeatability of the method in the optimized conditions were tested by intraday and interday tests; both showed a relative standard deviation lower than 10% for each analyte. Carryover effects were tested after each calibration curve, monitoring the presence of each analyte in a solution of water/acetonitrile 70:30. The recovery test was performed spiking three different raw tomato samples with a known amount of a mixture of APs and amino acids (final concentration 1µg/mL). The area of each peak of spiked sample was compared to the area count of a not spiked sample without any amino acid addition. Results confirmed the performance of HRMS: the detection of the defined *m/z* ratio turned out to be extremely accurate even if a complex mixture was injected, as a consequence the simplified extraction procedure surpassed the drawbacks due to matrix effect. The lowest recovery value of 85% was measured for cysteine, where some losses can be ascribed to the high reactivity of thiol group. A recovery of 105% was found for alanine and lysine, while for the five APs the recovery was 88%, 90% and 92% for fructosyl-asparagine, fructosyl-phenylalanine, fructosyl-histidine, respectively while for fructosyl-aspartic acid and fructosyl-lysine the recovery was 95%.

2.3.3 Amino acids and APs content in food samples

To verify the reliability of the method amino acids and APs content was monitored in two liquid matrices (UHT milk and low lactose milk), three semisolid matrices (raw tomato and thermal treated tomato) and a powdered sample (milk based caramel product). Furthermore, the method was used to analyze a canned tomato sampling which consisted in “Cherry” tomatoes (CT), “Datterini”

(YDT) and tomato paste (TP) and the effect of the thermal impact on tomato samples was illustrated by the FancyTyle approach recently developed [76].

Results on amino acids and APs are listed in **Tables S2.1** and **S2.2** of supplementary data while the ratio between APs and the sum of APs and free amino acids is reported in **Table 2.4** and it will be described and discussed in the following subparagraphs.

Table 2.S1: Amino acids concentration in the analyzed samples. The results were reported as μg/g of samples or μg/mL for milk. LL (low lactose milk); milk caramel based powder; T1, T2, T3 (raw tomatoes); CT (canned tomatoes), YDT (canned yellow "Datterini"), TP (tomato paste).

Amino acids	UHT milk	LLM	Milk caramel	T1	T2	T3	CT	YDT	TP
Ala	23.37 ± 0.20	11.89 ± 0.18	2.45 ± 0.04	234.34 ± 1.79	188.62 ± 1.63	310.51 ± 15.20	357.94 ± 12.12	106.53 ± 1.42	41.08 ± 0.93
Val	31.59 ± 0.19	37.29 ± 0.53	1.31 ± 0.05	5.14 ± 0.17	23.08 ± 1.70	28.56 ± 2.59	79.29 ± 1.26	32.88 ± 0.53	6.95 ± 0.05
Ile/Leu	1.13 ± 0.02	4.03 ± 0.12	5.35 ± 0.08	221.44 ± 0.28	241.23 ± 3.32	206.27 ± 1.90	153.56 ± 9.52	88.65 ± 1.72	33.62 ± 0.04
Phe	0.74 ± 0.04	2.87 ± 0.07	2.15 ± 0.04	211.65 ± 9.16	281.11 ± 1.78	404.52 ± 20.02	437.36 ± 8.96	451.53 ± 3.14	281.83 ± 0.08
Trp	0.10 ± 0.01	1.22 ± 0.02	0.17 ± 0.02	78.22 ± 0.89	59.06 ± 0.48	96.19 ± 1.87	245.24 ± 3.75	148.79 ± 3.31	57.49 ± 1.17
Tyr	0.22 ± 0.05	1.57 ± 0.03	0.41 ± 0.02	43.00 ± 0.14	2.80 ± 0.05	6.58 ± 0.23	106.87 ± 0.62	76.22 ± 1.48	91.95 ± 1.97
Cys	0.02 ± 0.001	0.02 ± 0.001	n.d	11.04 ± 0.15	6.61 ± 0.08	6.66 ± 0.75	0.40 ± 0.02	0.16 ± 0.01	0.13 ± 0.01
Met	n.d.	0.31 ± 0.01	0.003 ± 0.0001	3.49 ± 0.13	3.00 ± 0.09	3.09 ± 0.03	41.26 ± 0.07	25.09 ± 0.12	0.80 ± 0.03
Ser	1.43 ± 0.06	2.57 ± 0.06	1.71 ± 0.01	58.41 ± 0.30	51.80 ± 0.07	35.67 ± 5.43	94.99 ± 3.58	52.39 ± 0.95	24.53 ± 0.03
Thr	1.86 ± 0.56	2.16 ± 0.04	0.36 ± 0.01	118.62 ± 0.08	103.69 ± 1.32	105.19 ± 1.86	211.74 ± 7.27	68.67 ± 0.98	58.75 ± 0.32
Asn	5.85 ± 0.01	2.05 ± 0.01	0.54 ± 0.01	402.30 ± 0.92	336.78 ± 4.12	319.96 ± 5.53	1210.11 ± 9.31	215.47 ± 3.56	168.53 ± 2.64
Gln	1.67 ± 0.06	9.30 ± 0.20	0.001 ± 0.00001	2735.28 ± 2.93	2347.15 ± 29.13	2162.27 ± 121.00	10.48 ± 0.08	7.86 ± 0.02	0.16 ± 0.01
Asp	9.83 ± 0.08	8.27 ± 0.10	1.04 ± 0.01	703.03 ± 10.59	211.23 ± 3.85	202.80 ± 0.11	1596.96 ± 53.56	1151.36 ± 21.42	132.52 ± 0.40
Glu	159.89 ± 7.23	156.73 ± 1.28	0.42 ± 0.02	8002.18 ± 67.15	7008.68 ± 133.63	6077.83 ± 121.00	16646.89 ± 1385.40	12119.13 ± 96.86	1196.11 ± 53.09
Arg	12.76 ± 0.12	9.11 ± 0.21	2.25 ± 0.03	215.18 ± 0.89	213.82 ± 3.12	214.46 ± 2.74	218.38 ± 21.08	105.33 ± 2.72	170.13 ± 1.12
His	2.18 ± 0.17	0.99 ± 0.01	0.20 ± 0.02	440.94 ± 2.48	314.43 ± 4.73	404.21 ± 20.19	289.65 ± 6.01	147.77 ± 0.36	11.41 ± 0.33
Lys	16.77 ± 0.14	6.36 ± 0.03	47.15 ± 3.16	256.69 ± 1.57	315.13 ± 0.91	382.20 ± 6.77	226.06 ± 7.08	141.46 ± 3.01	64.97 ± 0.74
Pro	11.78 ± 0.02	11.41 ± 0.10	0.42 ± 0.02	17.61 ± 0.07	28.05 ± 0.55	19.53 ± 1.78	186.24 ± 3.33	93.56 ± 1.44	22.11 ± 0.65
Gly	22.86 ± 0.07	14.36 ± 0.02	0.10 ± 0.01	14.89 ± 0.01	11.22 ± 0.03	13.04 ± 2.86	27.04 ± 0.23	4.65 ± 0.03	6.77 ± 0.01

Table 2.S2: Amadori products concentration in the analyzed samples. The results were reported as μg/g of samples or μg/mL for milk. LL (low lactose milk); milk based caramel powder T1, T2, T3 (raw tomatoes); CT (canned tomatoes), YDT (canned yellow "Datterini"), TP (tomato paste).

Amadori products	UHT milk	LL milk	Milk caramel	T1	T2	T3	CT	YDT	TP
Fru-Ala	0.21 ± 0.001	19.69 ± 0.05	0.76 ± 0.02	4.60 ± 0.08	4.82 ± 0.1	9.71 ± 0.48	57.98 ± 0.13	37.25 ± 0.98	141.61 ± 0.51
Fru-Val	0.19 ± 0.001	1.42 ± 0.02	2.19 ± 0.01	2.49 ± 0.01	1.47 ± 0.18	2.20 ± 0.17	35.92 ± 0.53	29.90 ± 1.38	157.25 ± 0.45
Fru-Ile/Fru-Leu	0.10 ± 0.002	0.22 ± 0.01	1.29 ± 0.03	0.23 ± 0.03	0.043 ± 0.001	0.07 ± 0.003	45.03 ± 0.95	70.04 ± 2.40	12.37 ± 0.02
Fru-Phe	0.24 ± 0.001	0.44 ± 0.01	8.94 ± 0.07	0.53 ± 0.01	0.07 ± 0.001	0.19 ± 0.001	22.89 ± 1.94	75.74 ± 0.90	20.65 ± 0.08
Fru-Trp	0.12 ± 0.005	0.11 ± 0.01	2.56 ± 0.03	0.01 ± 0.001	0.08 ± 0.003	0.03 ± 0.001	7.17 ± 0.33	9.01 ± 0.29	35.36 ± 2.57
Fru-Tyr	0.05 ± 0.001	n.d.	1.99 ± 0.01	15.18 ± 0.39	11.15 ± 0.07	10.44 ± 0.31	38.65 ± 1.92	23.44 ± 1.46	63.52 ± 1.52
Fru-Cys	n.d.	0.11 ± 0.01	n.d	6.71 ± 0.05	0.89 ± 0.02	0.70 ± 0.01	16.50 ± 0.38	6.20 ± 0.42	0.87 ± 0.01
Fru-Met	n.d	n.d.	0.19 ± 0.01	0.07 ± 0.005	0.06 ± 0.001	0.06 ± 0.004	9.38 ± 0.38	14.29 ± 0.56	34.87 ± 0.12
Fru-Ser	0.36 ± 0.001	0.15 ± 0.01	0.41 ± 0.02	21.97 ± 1.88	30.13 ± 0.49	31.34 ± 0.47	34.63 ± 0.74	32.65 ± 0.84	20.14 ± 0.93
Fru-Thr	0.56 ± 0.010	0.52 ± 0.01	0.38 ± 0.02	15.79 ± 0.09	18.90 ± 0.4	27.10 ± 1.70	35.39 ± 0.70	27.70 ± 0.51	31.13 ± 0.13
Fru-Asn	0.11 ± 0.005	1.18 ± 0.01	1.49 ± 0.01	0.79 ± 0.03	1.85 ± 0.12	1.75 ± 0.02	115.18 ± 1.69	38.08 ± 1.14	55.12 ± 0.12
Fru-Gln	0.06 ± 0.001	0.72 ± 0.03	0.11 ± 0.01	3.11 ± 0.06	1.8 ± 0.08	1.88 ± 0.22	1.13 ± 0.14	1.25 ± 0.09	0.30 ± 0.01
Fru-Asp	0.41 ± 0.010	0.91 ± 0.01	1.37 ± 0.02	6.60 ± 0.24	1.70 ± 0.02	2.68 ± 0.11	383.69 ± 12.23	475.76 ± 12.30	128.73 ± 0.96
Fru-Glu	7.23 ± 0.050	25.12 ± 0.15	1.11 ± 0.02	43.18 ± 0.13	23.51 ± 0.25	32.55 ± 2.83	438.14 ± 2.89	731.77 ± 5.67	65.75 ± 0.01
Fru-Arg	0.12 ± 0.010	0.90 ± 0.03	2.45 ± 0.03	1.60 ± 0.01	0.03 ± 0.001	1.37 ± 0.05	63.29 ± 2.45	102.42 ± 0.33	774.82 ± 10.01
Fru-His	0.17 ± 0.003	0.80 ± 0.01	0.39 ± 0.02	0.05 ± 0.001	0.05 ± 0.003	0.17 ± 0.01	59.49 ± 6.90	70.02 ± 1.76	34.51 ± 0.19
Fru-Lys	2.41 ± 0.030	3.88 ± 0.09	84.31 ± 1.93	6.24 ± 0.01	3.28 ± 0.10	9.58 ± 0.16	90.93 ± 2.60	116.18 ± 3.31	48.81 ± 0.02
Fru-Pro	0.02 ± 0.008	0.12 ± 0.001	8.01 ± 0.06	0.04 ± 0.002	0.21 ± 0.02	0.13 ± 0.02	0.53 ± 0.12	0.60 ± 0.02	0.10 ± 0.01
Fru-Gly	0.07 ± 0.013	0.91 ± 0.02	0.04 ± 0.001	4.35 ± 0.05	3.88 ± 0.51	3.76 ± 0.03	4.38 ± 0.06	2.27 ± 0.10	2.10 ± 0.12

Table 2.4: Amadori products and amino acids ratio. The results were reported as (%) of free APs towards the sum of free APs and amino acids. For the concentration of each analytes in the different products see supplementary tables S1 and S2. LLM (low lactose milk); T1, T2, T3 (raw tomatoes); CT (canned tomatoes), YDT (canned yellow "Datterini"), TP (tomato paste).

Analytes Ratio	UHT Milk	LLM	Milk Caramel	T1	T2	T3	CT	YDT	TP
Ala	0.89	62.35	23.68	1.93	2.49	3.03	13.94	25.91	77.51
Val	0.60	3.67	62.57	32.63	5.99	7.15	31.18	47.63	95.77
Ile/Leu	8.13	5.18	19.43	0.10	0.02	0.03	22.67	44.14	26.90
Phe	24.49	13.29	80.61	0.25	0.02	0.05	4.97	14.36	6.83
Trp	54.55	8.27	93.77	0.01	0.14	0.03	2.84	5.71	38.08
Tyr	18.52	n.d.	82.92	26.09	79.93	61.34	26.56	23.52	40.86
Cys	n.d.	84.62	n.d.	37.80	11.87	9.51	97.63	97.48	87.00
Met	n.d.	n.d.	98.45	1.97	1.96	1.90	18.52	36.29	97.76
Ser	20.11	5.51	19.34	79.00	85.33	89.78	78.49	86.19	89.13
Thr	23.14	19.40	51.35	11.75	15.42	20.49	14.32	28.74	34.64
Asn	1.85	n.d.	73.40	0.20	0.55	0.54	8.69	15.02	24.65
Gln	3.47	7.19	99.10	0.11	0.08	0.09	9.73	13.72	65.22
Asp	4.00	9.91	56.85	0.93	0.80	1.30	19.37	29.24	49.27
Glu	4.33	13.81	72.55	0.54	0.33	0.53	2.56	5.69	5.21
Arg	0.93	8.99	52.13	0.74	0.01	0.63	22.47	49.30	82.00
His	7.23	44.69	66.10	0.01	0.02	0.04	17.04	32.15	75.15
Lys	12.57	37.89	64.13	2.37	1.03	2.45	28.69	45.09	42.90
Pro	0.17	1.04	95.02	0.23	0.74	0.66	0.28	0.64	0.45
Gly	0.31	5.96	28.57	22.61	25.70	22.38	13.94	32.80	23.76
Total ratio	**3.93**	**16.84**	**64.12**	**2.35**	**3.09**	**3.66**	**7.41**	**12.55**	**43.29**

- *Milk products*

The concentration of free amino acids in low lactose milk and UHT milk was roughly similar, as revealed by the ratio between the sum of free APs and the total amount of APs and amino acids. Some slight differences can be ascribed to the origin and the production processes. In UHT milk the ratio ranged from 0.17% for proline to 54.55 % for tryptophan and the total ratio was 3.93 %, while for low lactose milk it ranged from 1.04 % for proline up to 84.62 % for cysteine and the total ratio was 16.84 %. The concentration of glutamic acid was the highest, 159.89 ± 7.23 μg/mL in both milk products, while cysteine and methionine were almost absent. The concentration of aliphatic hydrophobic amino acids varied from 1.13 ± 0.02 μg/mL for isoleucine/leucine to 37.29 ± 0.20 μg/mL for valine in low lactose milk. Aromatic amino acids were particularly prone to the thermal oxidation and to the carbonyl attachment; their concentration was lower than 3 μg/mL. Polar neutral

amino acids varied from 1.43 ± 0.06 μg/mL for serine in UHT milk to 9.30 ± 0.20 μg/mL for glutamine in low lactose milk. Basic amino acids, such as arginine and lysine together with proline and glycine represented a good percentage of the total amino acids pool. Specifically the concentration of lysine was 16.77 ± 0.14 μg/mL and 6.36 ± 0.03 μg/mL for UHT milk and low lactose milk.

In milk products the concentration of APs was influenced by the presence of free amino acids and reducing sugars, even though an indirect relationship for the two groups was present for few analytes. The APs ranged from 0.10 ± 0.002 μg/mL for fructosyl-leucine/isoleucine in UHT milk to 25.12 ± 0.15 μg/mL for fructosyl-glutamic acid in low lactose milk. On one hand, in UHT milk, along with acidic amino acids, the overall extent of glycation was evident for fructosyl-glutamic acid (7.23 ± 0.05 μg/mL) and for polar basic fructosyl-lysine (2.41 ± 0.03 μg/mL). The concentration of the other APs was in the range 0.01 ± 0.001, 0.56 ± 0.01 μg/mL for fructosyl-leucine/isoleucine and fructosyl-threonine, respectively. On the other hand, in low lactose milk the concentration of APs varied between 0.11 ± 0.01 μg/mL for fructosyl-cysteine and fructosyl-tryptophan and 25.12 ± 0.15 μg/mL for fructosyl-glutamic acid revealing a direct effect of free glucose and galactose issued from hydrolysis of lactose.

In milk based caramel product lysine showed the highest concentration: 47.15 ± 3.16 μg/g, while the hydrophobic amino acids ranged from 2.15 ± 0.04 μg/g to 5.35 ± 0.08 μg/g for phenylalanine and isoleucine/leucine respectively. Interestingly, the highly reactive amino acids were influenced by the production process and their concentration varied from 0.001 ± 0.0001 μg/g for glutamine or 0.003 ± 0.00001 μg/g for methionine to 2.25 ± 0.03 μg/g for arginine. Cysteine was not detected likely due to the reactivity of thiol group.

Fructosyl-lysine was 84.31 ± 1.93 μg/g, indicating that the severe thermal treatment determined an extensive lysine blockage. It was the most abundant APs in milk based caramel, followed by fructosyl-proline and fructosyl-phenylalanine, whose concentrations were 8.01 ± 0.06 μg/g and 8.94 ± 0.07 μg/g, respectively. As highlighted in **Table 2.4** the ratio of the two analytes classes showed the highest value among the analyzed products: 64.12%. Despite a concentration of free amino acids lower than UHT and low lactose milk, the total amount of APs was two and nine times higher than UHT and low lactose milk, respectively. The ratio ranged from 19.34% for serine and 99.10 % for glutamine indicating that the thermal treatment can be linked to the MR extent and other MRPs formation, mainly flavor compounds [37, 77].

- *Tomato products*

In raw tomato samples the ratio between APs and the sum of APs and free aminoacids was almost similar among the three cultivars, with some exceptions for valine and arginine; it ranged from 0.01 for arginine in T2, histidine and tryptophan in T1 and 79.93 % for tyrosine in T2. The total ratio was 2.35 %, 3.09 % and 3.66% for T1, T2 and T3 respectively. On the opposite, in processed tomatoes the total ratio was 7.41%, 12.55% and 43.29% for canned tomatoes, "Datterini" and tomato paste, respectively. In tomato samples, free glutamic acid showed the highest concentration in raw tomatoes ranging from 6077.83 ± 121.00 to 8002.18 ± 67.15 μg/g FW. The amount of other amino acids varied according to the different cultivars: specifically the concentration of acidic amino acids such as glutamic acid and aspartic acid were higher in T1 than T2 and T3 and those were the highest values among the amino acid pattern, while lysine ranged from 256.69 ± 1.57 to 315 ± 0.91 and 382.20 ± 6.77 μg/g FW in T1, T2 and T3 respectively. The concentration of valine was the lowest among the hydrophobic amino acids (from 5.14 ± 0.17 to 23.08 ± 1.70 μg/g FW), while polar neutral amino acids were mainly characterized by the presence of asparagine and glutamine whose concentration was higher than 300 μg/g FW and 2150 μg/g FW, respectively Three different categories of thermal treated products were analyzed in order to evaluate not only the concentration of amino acids, but also to explore the link between free amino acids, APs, MR and thermal treatments. The concentration of glutamic acid in these products was about three times higher than in raw tomatoes: it varied from 1196.1 ± 53.09 to 16646.89 ± 1385.40 μg/g FW.

The concentration of APs in tomato samples was influenced by the concentration of free amino acids, reducing sugars, harvesting procedure, ripening and last but not the least by the thermal processes. In raw tomatoes, APs concentration was quite constant among the three cultivars, even if some exceptions can be linked to the above mentioned variables. In general APs concentration ranged from 0.01 ± 0.0001 μg/g for fructosyl-tryptophan to 43.18 ± 0.13, 27.10 ± 1.70, 31.34 ± 0.47 and 15.18 ± 0.39 for fructosyl-glutamic acid, fructosyl-threonine, fructosyl-serine and fructosyl-tyrosine, respectively. As expected the values of APs in thermal treated tomatoes were higher than in raw tomatoes. The concentration of APs derived from hydrophobic amino acids in canned Cherry tomatoes was higher than in tomato paste, varying from 12.37 ± 0.02 μg/g for fructosyl-leucine/isoleucine in tomato paste to 37.25 ± 0.98 μg/g for fructosyl-alanine in yellow canned "Datterini". The concentration of fructosyl-glutamic acid, fructosyl-lysine and fructosyl-serine in tomato paste were 67.25 ± 0.01, 48.81 ± 0.02 and 20.14 ± 0.93 μg/g, respectively.

In **Table 2.5** the differences among the samples were shown using a visual tool, named FancyTile [76]. Up to now this representation was based on semi-quantitative data while here the scheme was constructed using the concentrations of nine amino acids. The FancyTile immediately highlights the impact of the thermal treatment on three products, namely canned Cherry tomatoes, canned yellow "Datterini" and tomato paste. Tomato paste showed the most severe thermal treatment: the free amino acid FancyTile showed all blue squares indicative of a low amount of free amino acids, while the free amino acid image of canned yellow "Datterini" and canned Cherry tomatoes had mostly green marked and yellow/orange tiles indicative of greater amount of them.

In **Table 2.6** FancyTile representation was applied using the concentration of nine APs. On one hand, tomato paste showed a dominant green color respect to the other two canned products confirming that the extent of glycation was more intense on this sample. On the other hand, not all the amino acids have the same trend: most of them and particularly fructosyl-alanine had the highest concentration in the tomato paste, fructosyl-lysine and fructosyl-asparagine had higher concentration in "Datterini" and in Cherry tomato, respectively, suggesting that in these samples the MR further progressed probably leading to the formation of flavor compounds and other products such as HMF, furan and dicarbonyls [78, 79].

Table 5: Amino acids FancyTiles in thermal treated tomatoes. The quantitative colorimetric scale was reported in µg/g.

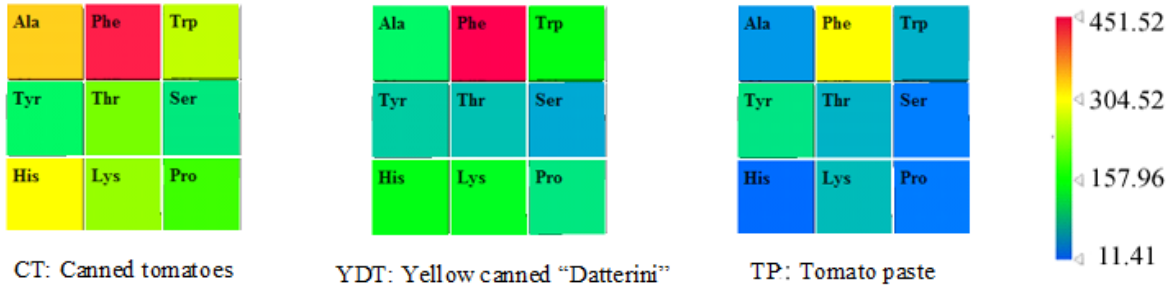
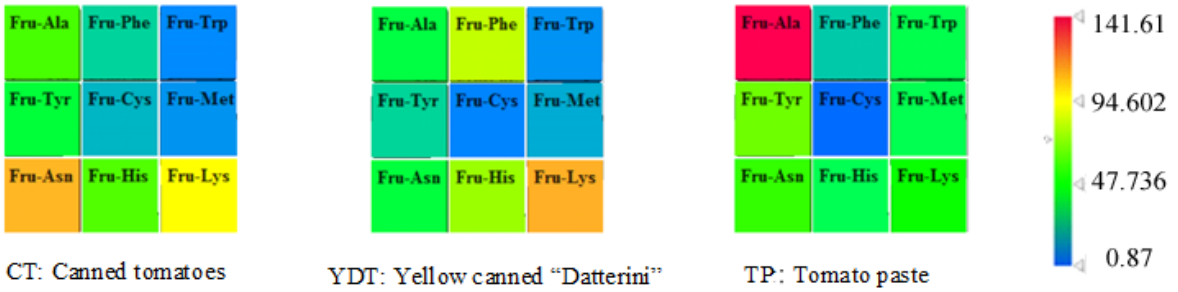


Table 6: Amadori products FancyTiles in thermal treated tomatoes. The quantitative colorimetric scale was reported in µg/g.



The present paper represents one of the first examples of simultaneous detection of amino acids and APs. The conventional reversed phase C18 and C8 column were not sufficient to ensure the separation between free amino acids and their respective APs counterparts and the results were unsatisfactory [65]. Previous papers reported the separation of APs with the use of ion exchange column or an ion exchange with electrochemical detection coupled to post-column addition of NaOH [60, 61, 80], with HILIC phase [81], on a bare HILIC silica column at high temperature [82] and by ion pairing through heptafluorobutyric acid [83].

In the present study the use of an ion pairing agent, such as NFPA, definitively separated the peaks of APs from free amino acids, even if it is still not possible to separate APs from their corresponding Schiff bases; the two compounds have exactly the same *m/z* in positive ions and in most cases the same chemical and fragmentation behavior. It derives that the chromatographic profile is the same thus, their accurate detection and quantitation is difficult.

The co-elution was avoided in order to prevent the simultaneous ionization of reducing sugars, APs and amino acids and the consequent overestimation or underestimation of the signal associated to the target compounds. The shapes of the peaks and the retention time among the other chromatographic performances were ensured by the core shell materials of the chromatographic column adopted [84]. Moreover the use of a core shell column allowed to set the instrumental resolution to 50000 (FWHM, *m/z* 200) and to gain a good compromise between number of ions injected (balanced mode) and scan time. The exact mass of diisooctyl phthalate, one of the most common contaminants in mass spectrometry [85], was used as internal mass reference in order to allow the recalibration of the instrument. By this strategy the mass analyzer can correct each mass trace according to the detection of the *m/z* 391.28429. The result was the increase of the mass accuracy of each target compound. The same approach was followed in previous papers by our group [75, 76].

Looking at the different matrix it is possible to highlight the main advantages of the proposed method. Using the developed procedure, free amino acids and APs were measured in different foods in order to have preliminary information on the possibility of evaluating the importance of APs as a direct marker of the quality of the starting material and of the thermal impact. On one hand, the available literature data on the quantification of single amino acids in foods usually refer to the concentrations that can be measured after protein hydrolysis. As a consequence working only with free amino acids allowed to rule out the main drawbacks due to the chemical transformation caused

by the severe acidic treatment. They are the conversion of asparagine and glutamine into aspartic acid and glutamic acid; the sulphoxidation of the thioether side chain of methionine, the modification of the phenolic group of tyrosine and phenylalanine: the degradation of the indole ring of tryptophan; the esterification reaction of tyrosine and serine with glutamic acid [86].

The assessment of thermal damage on free amino acid is based on the assumption that the formation of APs on free amino acids is well representative of the derivatization occurring at the side amino group of the amino acids present in the proteins. The results obtained in this study confirmed this hypothesis: in fact, the extent of amino acid modifications observed considering only the free amino acids moiety are well in line with those previously observed measuring amino acid derivatization after protein hydrolysis. This was verified, in particular, for the UHT milk sample where many data are available [58].

Investigating the concentration of lysine in milk samples was particularly useful to deepen the aspect linked to Nε-(carboxymethyl)-lysine (CML) and Nε-(carboxyethyl)-lysine (CEL) formation: data of this paper showed that lysine represented about 5 % of the total free amino acids which is similar to the percentage obtained after acidic hydrolysis on total protein [87, 88]. The results were in good agreement with those obtained earlier in infant formula confirming the performances of the developed method in a complex matrix such as milk [51, 89, 90].

Because of the presence of glucose and galactose, low lactose milk showed a higher amount of APs than UHT milk for all compounds, with the only one exception represented by fructosyl-serine and fructosyl-threonine. Taking into account the different thermal treatments and storage condition, the differences between UHT milk and low lactose milk were particularly evident for aliphatic APs such as fructosyl-alanine and fructosyl-valine, and for acidic APs such as fructosyl-glutamic acid while for fructosyl-lysine the two concentrations were almost comparable. Specifically this last compound was directly implicated in the formation of CML and CEL [91]. The reported values for fructosyl-lysine were higher than the one reported earlier [92] and these differences can be related not only to the extraction procedure, but also to the acidic hydrolysis performed for the quantitation of protein-bound fructosyl-lysine. On the contrary, the concentration of fructosyl-lysine here reported was lower than the one calculated through the furosine method after acidic hydrolysis highlighting some discrepancies between the direct and indirect analytical methods [93]. High resolution mass spectrometry revealed a new scenario in the monitoring of the early stage of MR overcoming the drawbacks linked to acidic hydrolysis or to enzymatic digestion [58, 86]. Although,

for a complete picture of the milk thermal damage the measure of lactosylated derivatives would be necessary.

Some interesting points can be highlighted from the data on milk based caramel and from the behavior of lysine in the three products. As expected, the overall amount of amino acids was around four times higher in UHT and low lactose milk than in caramel powder, while the derivatization ratio was very high (64.12%) likely due to the severe thermal load and to the abundance of reducing sugars and dicarbonyls. The derivatization rate of lysine jumped from 12.57% and 37.89% in UHT and low lactose milk respectively to 64.13% in caramel powder highlighting the direct effect of carbonyl groups on amino moiety.

Tomato products, mainly dried tomatoes, are one of the most studied food as far as the formation of APs [94]. Free amino acid concentrations in raw tomatoes are highly variable: the differences can be ascribed to several factors such as variety, cultivar, ripening, storage [51, 95, 96]. This is confirmed by our results which are in some cases in line with those previously reported and in others very different. Our values are of the same order of magnitude towards previously reported papers ranging from 0.13 ± 0.01 μg/g for cysteine in tomato paste to 16646.89 ± 1385 for glutamic acid in canned "Cherry" tomatoes. Specifically, the concentration of amino acids in tomato paste, lower than canned tomatoes, can be related to the Maillard cascade, where the intermediate stage favored the dehydration, oxidation, fragmentation, and other reactions of the Schiff bases of basic and polar amino acids. The final stage of the Maillard reaction is characterized by the formation of stable volatile components, cross-linked products, mainly proteins, and aromatics and colored polymers, such as melanoidins. The concentration of glutamic acid was six and nine times lower than the ones revealed canned yellow "Datterini" and canned tomatoes, respectively. The concentration of the other amino acids in tomato paste was lower than in canned tomato, with the only exception being tyrosine (91.95 ± 1.97 μg/g FW) and glycine (6.77 ± 0.0 μg/g FW) towards canned "Datterini". According to the high reactivity of side chain, the thermal damage was particularly pronounced on lysine, glutamic acid, proline, methionine and alanine whose values were lower compared to those from canned tomatoes of almost three, eight, eight, fifty and seven times, respectively.

Fructosyl-glutamic acid in canned tomato was the most abundant APs and the concentration here reported was significantly different from that reported for dried tomatoes and fruits and this can be

due to the differences in the raw material and to the thermal process employed [61, 67, 94]. It is followed by fructosyl-aspartic acid, fructosyl-asparagine and fructosyl-lysine.

The relevance of this paper can be evaluated also through the ratio between APs and the sum of APs and amino acids in tomato products that varied according to thermal treatment performed (**Table 2.4**): it ranged from around 3% in raw tomatoes to 7.41%, 12.55% and 43.29% for "Cherry" tomatoes, canned "Datterini" and tomato paste. In particular, in the thermal treated products the effect of thermal load was impressive for thermo labile amino acids such as tryptophan (2.84 %, 5.71% and 38.08 %), methionine (18.52 %, 36.29 % and 97.76 %) and histidine (17.04 %, 32.15 %, 75.15 %).

The meaning of the results related to the free amino acids and corresponding APs in tomatoes were summarized in the two FancyTiles of **Table 2.5** and **2.6**. Even if the starting materials and the thermal treatments were different, results suggested that histidine, alanine and lysine were particular sensitive parameters, while the aromatic phenylalanine underwent the highest reduction percentage. These results gave a preliminary overview on the potentiality related to the monitoring of free amino acids and APs, whose ratio can also be an interesting aspect for the evaluation of the starting raw material, of the general thermal impact and of the final quality not only in the hereby analyzed samples, but also in milk based products, fruit juices, bread, biscuits, French fries and so on.

In conclusion, the present paper highlights the possibility to simultaneously quantify free amino acids and their respective APs following a simplified approach that avoids the extraction protocols or derivatization procedures. Moreover, the quantification of APs in several food matrix represents the key that may allow to skip complex, time consuming and expensive procedures linked to the acidic hydrolysis of proteins and to the derivatization procedures. By monitoring the ratio between free amino acids and APs there is the opportunity to create a deep fingerprinting of the quality of the starting material, of the thermal treatment and of the extent of the MR in the initial steps.

Chapter 3

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

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Abstract

The control of Maillard reaction (MR) is a key point to ensure processed foods quality. Due to the presence of a primary amino group on its side chain, lysine is particularly prone to chemical modifications with the formation of Amadori products (AP), Nε-(carboxymethyl)-L-lysine (CML), Nε-(carboxyethyl)-L-lysine (CEL). A new analytical strategy was proposed which allowed to simultaneously quantify lysine, CML, CEL and the Nε-(2-furoylmethyl)-L-lysine (furosine), the indirect marker of AP. The procedure is based on stable isotope dilution assay followed by, liquid chromatography tandem mass spectrometry. It showed high sensitivity and good reproducibility and repeatability in different foods. The limit of detection and the RSD% were lower than 5 ppb and below 8%, respectively. Results obtained with the new procedure not only improved the knowledge about the reliability of thermal treatment markers, but also defined new insights in the relationship between Maillard reaction products and their precursors.

Keywords: Maillard reaction, LC-MS/MS, CML, CEL, lysine, furosine

3.1 Introduction

The final quality of many industrial food products depends on food formulation and processing design resulting in the formation of a huge variety of molecules as a consequence of thermal treatments and chemical changes [97]. Along with lipid oxidation, the Maillard reaction (MR) occupies a prominent place in the final quality of foods being responsible not only for the desired color and aroma compounds but also for the formation of potentially toxic Maillard reaction end products (MRPs). The reaction between reducing sugars and amino groups is the first step in the Maillard cascade: the formation of the stable 1-amino-1-deoxy-2-ketose the Amadori product (AP) and 2-amino-2-deoxyaldose Heyns products represents the starting point of the many chemical pathways of this reaction [98]. The presence of an amino group on the side chain of lysine makes this amino acid particularly sensitive to the carbonyls attachments. The modifications arising from the lysine blockage resulted in the formation of a bewildering array of molecules: Nε-(1-Deoxy-D-fructos-1-yl)-L-lysine (fructosyl-lysine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL), pentosidine, pyrraline, lysino-alanine, 5-hydroxymethylfurfural (HMF), α-dicarbonyls and aroma key odorants [13]. Fructosyl-lysine, CML and CEL represent the most widely studied MRPs, and they are often used as biomarker of food quality [99, 100]. As highlighted in **Figure 3.1**, the acid hydrolysis adopted to release free amino acids from the polypeptide chain promote the conversion of the 1-deoxy-fructosyl-L-Lysine (AP) through a cyclized Schiff base, into the of Nε-(2-furoylmethyl)-L-lysine (furosine) which is a compound that can be quantified after protein hydrolysis and it has been widely used as marker of thermal treatment particularly in the dairy products [101].

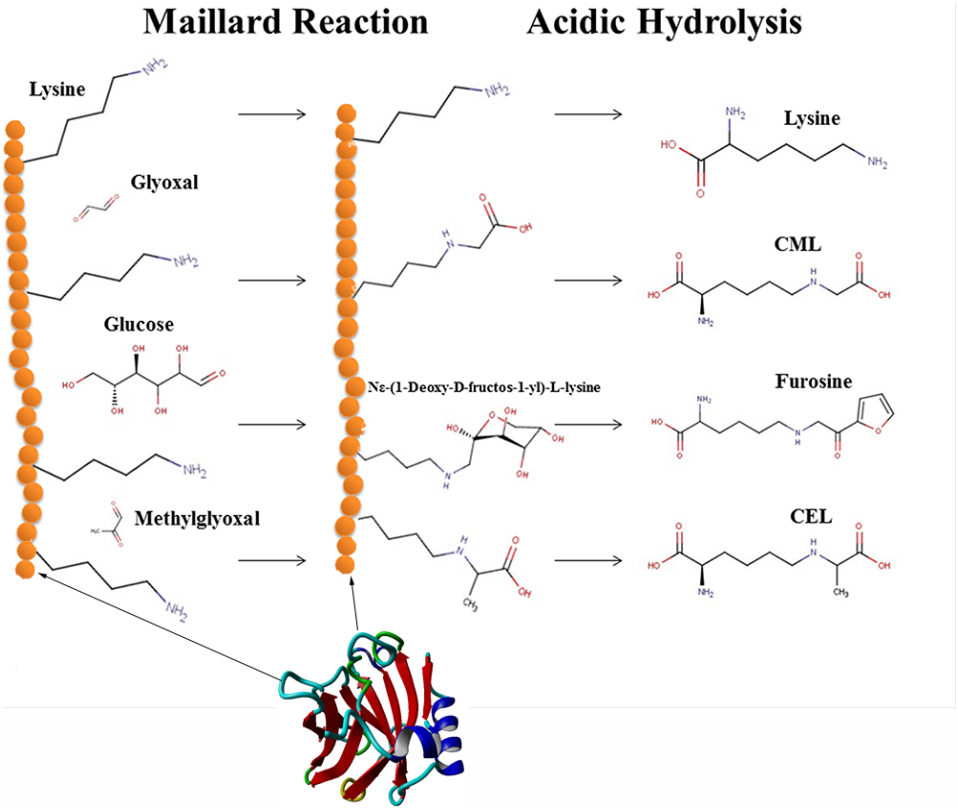

Figure 3.1: Effect of glucose and dicarbonyls on the formation of protein-bound MRPs. At the bottom the structure of β-Lactoglobulin [102].

The formation of CML and CEL from the oxidation of ARP and HRP has been well characterized [99]. Carbohydrate fragmentation allows the formation of glyoxal and methylglyoxal that readily react with lysine residues yielding the glycoxidation products CML and CEL, respectively [103]. Moreover, CML and CEL can be formed via the Namiki-pathway through three subsequent steps: Schiff base production, glycolaldehyde alkylimine synthesis, oxidation and formation of glyoxal or methylglyoxal which react with lysine to yield CML and CEL. Another route of CML and CEL formation is linked to lipid peroxidation as glyoxal and methylglyoxal can derive from polyunsatured fatty acids [6]. Moreover, the two markers can be also formed from fragmentation and subsequent glycation of ascorbic acid and dehydroascorbic acid [104].

From the analytical point of view the identification of these markers of heat treatment can be approached in several ways [105]. Furosine is used as indirect marker of quality control of moderately heat-treated dairy samples. The golden standards for furosine detection are ion-exchange chromatography, reverse phase high performance liquid chromatography (RP-HPLC) with UV detection, [57] capillary electrophoresis and ion-pairing HPLC by using sodium-

heptanosulphonate [106]. These procedures had several drawbacks mainly related to the modifications occurring during sample preparation: the acidic hydrolysis does not allow the differentiation between AP and glycosyl-amine; overestimation or underestimation linked to the acidic hydrolysis might occur due to the formation of further intermediates and end-products [58].

As for furosine, CML and CEL analysis implies acidic hydrolysis to hydrolyze peptide bonds followed by their quantification that could be performed by different instrumental methods [99]. In some papers a pre-column derivatization with o-phthalaldehyde was used to allow the detection by florescence detector [107], while a widely used approach for CML and CEL detection is gas or liquid chromatography coupled with tandem mass spectrometry. Specifically, multiple reaction monitoring (MRM) mode improves the sensitivity, reduces the coefficient of variability and ruled out the problems of derivatization [108]. A double derivatization is required for GC separation and this bottleneck highlights the advantages of LC-MS/MS detection: no derivatization, highest sensitivity and good reproducibility [109, 110]. Moreover CML, CEL and lysine detection is possible also by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) that allows relative quantification of protein lactosylation and it is a reliable method to monitor the early Maillard reaction as well as MRPs during milk processing [111].

The aim of the present paper, was to further improve the existing methodologies for the detection of lysine and MRPs. A new method was designed which included direct hydrolysis along with stable isotope dilution assay coupled with solid phase extraction and ion pairing liquid chromatography tandem mass spectrometry (LC-MS/MS). The developed procedure allowed the simultaneous detection of total lysine, furosine, CML and CEL. The method was tested on several foods: milk, infant formulas, cookies, bread slices. The robustness after several injections and the reliability of the results obtained were evaluated in soybean-based feed products obtained under severe thermal treatment conditions. Data demonstrated satisfactory analytical performances on all tested samples and results were perfectly in line with those previously obtained.

3.2 Material and methods

3.2.1 Chemicals and reagents

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%) and the analytical standards L-lysine hydrochloride

and [4,4,5,5-d_4]-L-lysine hydrochloride (d_4-Lys) 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-(Carboxy[$^2H_2$]methyl)-L-Lysine (d_2-CML) were obtained from Polypeptide laboratories (Strasbourg, France), Nε-(Carboxyethyl)-L-lysine and its internal standard Nε-(Carboxy[$^2H_4$]ethyl)-L-lysine (d_4-CEL) were purchased from TRC-Chemicals (North York, Canada).

3.2.2 Foods samples

Powdered infant formula and milk samples were purchased in a local market, biscuits samples and bread slices were prepared according previous papers published by our group [112, 113]. UHT milk was prepared according to the procedure previously described [114]. Raw milk (protein, 3.5%; fat, 1%) was purchased in a local market.

3.2.2.1 Soybean samples

One batch of quartered raw soybeans was purchased from Rieder Asamhof GmbH & Co. KG (Kissing, Germany).The raw soybeans were further processed at the hydrothermal cooking plant of Amandus Kahl GmbH & Co. KG (Reinbeck, Germany). First, the beans were short-term conditioned to reach a temperature of 80 °C after 45 seconds. Afterwards, the beans entered a hydrothermic belt cooker at 72 °C and left inside for 3 min at a temperature of 70 °C. Then they were expanded at 117 °C using an annular gap expander (Typ OEE 8, Amandus Kahl GmbH & Co. KG, Reinbeck, Germany). The expanded soybeans were collected in a drying wagon for 10 min. Then they were dried with air at 65 °C for 10 min and cooled for another 10 min to reach a final moisture content of 12%. Afterwards, the expanded soybeans were autoclaved for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 60 min at 110 °C and 1470 mbar using a fully controlled autoclave (Typ HST 6x9x12, Zirbus Technology GmbH, Bad Grund, Germany).

3.2.3 Samples preparation

Lysine and its derivatives Nε-(2-Furoylmethyl)-L-lysine (furosine), Nε-(Carboxymethyl)-L-lysine (CML), Nε-(Carboxyethyl)-L-lysine (CEL) were analyzed considering previous papers [9, 108, 109] and introducing several modifications. Briefly, 100 mg of each sample was accurately weighed in a screw capped flask with PTFE septa and 4 mL of hydrochloric acid (6 N) was added. The mixture was saturated by nitrogen (15 min at 2 bar) and hydrolyzed in an air forced circulating oven

(Memmert, Schwabach, Germany) for 20 h at 110° C. The mixture was filtrated by polyvinylidene fluoride filters (PVDF, 0.22 Millipore, Billerica, MA) and 400 µl was dried under nitrogen flow in order to prevent the oxidation of the constituents. The samples were reconstituted in 370 µl of water and 10 µL of each internal standard d_4-Lys, d_2-CML and d_4-CEL was added in order to obtain a final concentration of 200 ng/mg of samples for both standards. Samples were loaded onto equilibrated Oasis HLB 1 cc cartridges (Waters, Wexford, Ireland) and eluted according to the method previously described, then 5 µl was injected onto the LC/MS/MS system.

3.2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Separation of furosine, CML, CEL, lysine and their respective internal standards was achieved on a reversed – phase core shell HPLC column (Kinetex C18 2.6 µm, 2.1 mm x 100 mm, Phenomenex, Torrance) using the following mobile phases: A, 5 mM perfluoropentanoic acid and B, acetonitrile 5 mM perfluoropentanoic acid. The compounds were eluted at 200 µL/min through the following gradient of solvent B (t in [min]/[%B]): (0/10), (2/10), (5/70), (7/70), (9/90), (10/90), (12/10), (15/10). 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, cad gas and curtain gas were set to 45 and 5 (arbitrary units). The chromatographic profile was recorded in MRM mode and the characteristic transitions were monitored in order to improve selectivity using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). All relevant parameters are summarized in **Table 3.1**.

Table 3.1: Mass spectrometry set up

Compounds	$[M+H]^+$	Fragments	CE (V)	DP (V)
CML	205	84	29	30
		130.2	27	30
***d_2*-CML**	207	84	30	20
		144	21	20
		130	17	20
Furosine	255.1	130	18	21
		84.4	28	21
Lys	147.2	130.2	16	30
		84.1	24	30
***d4* –Lys**	151.3	134.1	15	30
		88.2	26	30
CEL	219.2	130.3	20	30
		84.0	28	30
***d4*-CEL**	223	134.1	18	25
		88.0	30	25

3.2.5 Analytical performances

CML, CEL, furosine and total lysine were quantified using a linear calibration curve built with specific solutions of CML spiked with d_2-CML, lysine and furosine spiked with d_4-lysine and CEL spiked with d_4-CEL (final concentration of internal standards: 200 ng/ml) dissolved in water. The limit of detection (LOD) and the limit of quantitation (LOQ) were monitored according to the signal to noise ratio [115]. The coefficients of determination r^2 for the 4 analytes were tested plotting the ratio between the pure compounds and their respective internal and the concentration of the pure compounds in the linearity range 5-1000 ng/mL. The internal standard ratio was used for the quantification and the relative standard deviation of intraday and interday assay was monitored three times each day and six times in different days. The recovery test was monitored according to the concentration of the internal standards used and to the ratio between labeled compounds and native compounds.

3.2.6 Statistical analysis

All of the analyses were performed in quadruplicate and the results expressed as mg/100 g of protein. Statistical calculations were performed using Matlab R2009b (Natick, MA) while for mass spectrometry data, Analyst version 1.4.2 (Applied Biosystems, Carlsbad, CA) was used.

3.3 Results and discussion

3.3.1 Liquid chromatography set up

Under the above described chromatographic conditions, typical retention time of CML and d_2-CML was 7.11 min, for d_4-Lys and Lys it was 7.23 min, for furosine it was 7.91 min, while for CEL and d_4-CEL it was 7.36 min (**Figure 3.2**). Previous papers highlighted the problems due to the poor retention of amino acids and their derived molecules on silica bonded and C-18 column [83]. Preliminary trials performed using C-18 column without the ion pairing agent confirmed this feature: the retention was poor and the analytes co-eluted with the impurities on the front of the chromatographic run with the consequent partial suppression of the signal associated to the markers. Inadequate separation of the analytes was obtained also using polar end-capped column; however a significant improvement was obtained using with this column perfluoropentanoic acid as ion pairing agent. In these experimental conditions, the retention time followed a typical reversed phase profile according to the polarity and to the steric hindrance of each molecule, as previously observed by

other papers published earlier [109, 116]. The presence of the ion pairing agent charged the core shell residues increasing the retention and promoting the selectivity of the positively charged CML, CEL, furosine, lysine and their respective internal standards. The presence of a core shell phase increased of the resolution which directly reflects the good performances of the reported method, the shape of the peak was maintained over each batch and the retention time shift was lower than 0.5 min, highlighting the robustness of the analytical performances.

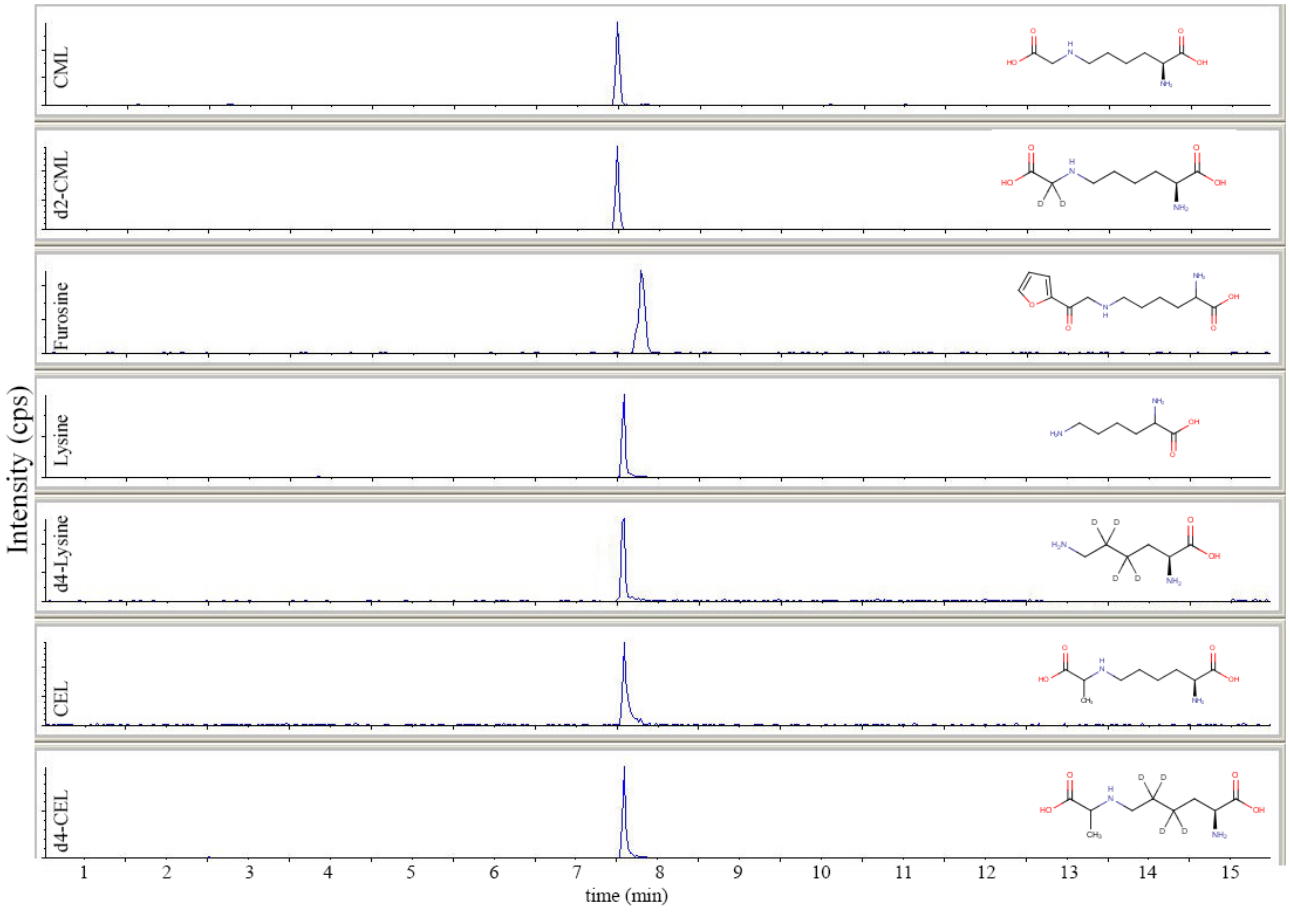

Figure 3.2: Extracted ion chromatogram of the four target molecules and their respective internal standards

3.3.2 Mass spectrometry set up

Mass spectrometry conditions were optimized by infusing singularly the seven standards directly in the ion source. Collision energy, declustering potential, tube lens voltage along with spray voltage and interface temperature were monitored in order to favor the formation of the typical fragmentation pattern [108]. The lysine derived compounds underwent the formation of the

fragment ion at 130 *m/z* which corresponds to the pipecolic acid generated by the subsequent cyclization of the side chain of lysine and the loss of ε- amino group, similarly the mass shift for deuterated standards *d4*-CEL and *d4*-Lys was +4 Da as consequence of the fragmentation occurred on the side chain of lysine (**Figure 3.S1** in supplementary material section) [117]. The MRM revealed the loss of formic acid giving the typical fragment at *m/z* 84; the mass shift for the deuterated molecules was +4. The seven standards were also infused inside the chromatographic flow in order to evaluate the interferences due to the ion pairing agent or to the solvent and the results revealed that no enhancement or suppression effect can be ascribed to the parameters monitored.

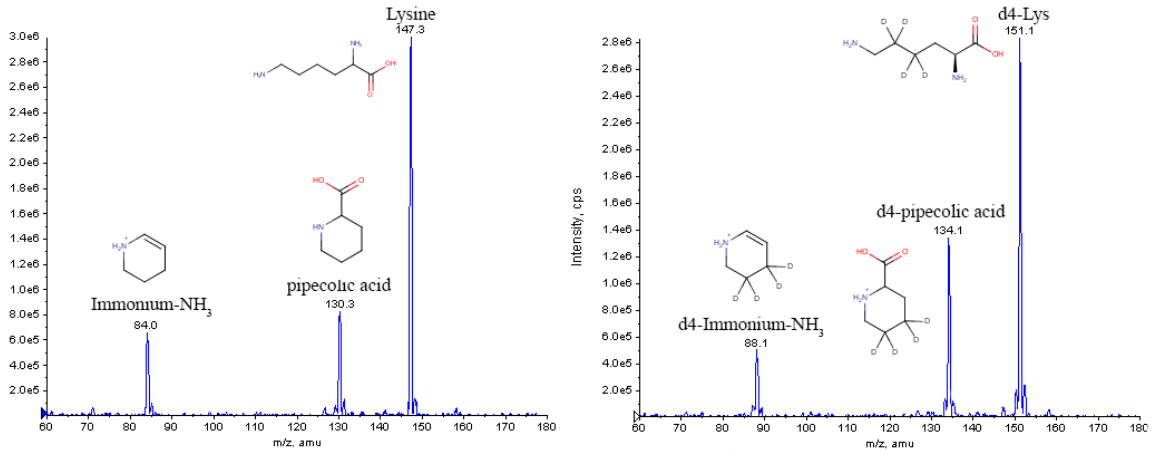

Figure 3.S1: Fragmentation pathway for lysine and its deuterated internal standard *d4*-lysine. The structures of pipecolic acid and 1,2,3,4-tetrahydropyridin-1-ium ion was reported [117].

3.3.3 Analytical performances

The analytical performances of the method were tested against reproducibility, repeatability, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, carry-over and coefficient of correlation (r^2). Before and after each batch, three solutions of acetonitrile and water (90:10; 50:50 and 10:90) were injected in order to verify the absence of any contaminants with the same signal and the same retention time of the analyzed molecules. The limit of detection and the limit of quantitation were determined according to the procedure previously described. The concentration 0.1 ppb resulted in no signal, while the LOD was 0.5 ppb for CML and lysine while for CEL and furosine it was 1 and 3 ppb, respectively. The slight differences among CML, CEL and furosine can

be related to the different stability in the injection conditions. By injecting these concentrations the signal to noise ratio was always higher than 3. The LOQ were 5 ppb for CML, CEL and Lysine while for furosine it was 9 ppb, as highlighted in **Table 3.2**. These values were perfectly in line with those previously described for CML, CEL and lysine quantification by MS/MS [108, 118] while for furosine the performance of LOD and LOQ were below the values previously reported in milk [119]. According to the LOD and LOQ, linearity was achieved in the range 5-1000 ppb for CML, CEL and lysine, while for furosine the linearity range was between 9 and 1000 ppb. The carryover effect was tested injecting after each point of the calibration curves a solution consisting in acetonitrile and water (50:50, v/v) and verifying the absence of the target compounds. The linearity of the calibration curves was evaluated three times in the same day (intraday assay for the reproducibility) and three times for three subsequent days (interday assay for the repeatability) using the ratio between the target compounds and their respective internal standard. The RSD (%) among the three curves was always lower than 8%, demonstrating that external factors had marginal impact on the performance of the method. Each point of the calibration curves was monitored using two specific transitions: the most intensive fragment was used as quantifier, the lowest as qualifier. For CML, CEL, furosine and lysine, the respective transitions of *m/z* 205−84.1, *m/z* 219.1−84.1, *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* 219.1−84.1, *m/z* 255.1−84, and *m/z* 147.2−84.1 were used as qualifier. CML was quantified using *d2*-CML as internal standard (*m/z* 207−144.1 and 207−84 for quantification and confirmation, respectively), CEL was quantified using *d4*-CEL (*m/z* 223−134.1 and 223 − 84 for quantification and confirmation, respectively) whereas for furosine and lysine, *d4*-lysine was used (*m/z* 151.2−134.1 and *m/z* 151.1− 88 for quantification and confirmation, respectively). The use of *d4*-lysine as internal standard for the quantification and recovery of furosine was optimized by monitoring the relative intensity of furosine standard towards *d4*-CEL, *d2*-CML and *d4*-lysine. A mixture of the four standards (10 ppm) was directly infused in the ion source. Results revealed that the intensity of the signal at *m/z* 151.2 and *m/z* 255 were similar and both were 15% higher than the signal of *d2*-CML and *d4*-CEL.

Table 3.2: Analytical performances for the four analytes and their respective internal standards

Compound	LOD	LOQ	RSD [%]	Linearity range	r^2	Recovery
CML	0.5 ppb	5 ppb	7	5-1000 ng/ml	> 0.99	91.1 ± 8.4
CEL	1 ppb	5 ppb	5	5-1000 ng/ml	> 0.99	84.2 ± 7.4
Lysine	0.5 ppb	5 ppb	5	5-1000 ng/ml	> 0.99	88 .0± 6.9
Furosine	3 ppb	9 ppb	8	9-1000 ng/ml	> 0.99	88.0 ± 6.9

The response of the method in food was tested during each batch evaluating the ratio between the target compounds and the internal standard, these procedures confirmed and deepened the aspects linked to the recovery assay: in each sample the ratio between the area of the analyte and the area of the deuterated compounds was compared towards the calibration curve in order to obtain the final concentration of the analytes in the matrix. The intensity of the internal standard in the samples and in the standard was compared and the RSD (%) between the spiked samples and the spiked standards was always lower than 10%. The recovery test was monitored in all the food matrix according to the intensity of the internal standard, the results were 91.1 ± 8.4, 84.2 ± 7.4, 88.0 ± 6.9 for *d2*-CML, *d4*-CEL and *d4*-Lysine.

3.3.4 CML, CEL, furosine and total Lysine in food

Powdered samples were freeze dried prior analysis in order to remove the interferences due to the humidity. The extraction procedure of MRPs is characterized by three key steps: the reduction with sodium borohydride, the hydrolysis with hydrochloric acid and the stable isotope dilution assay prior ion pairing solid phase extraction. According to the nature of protein and to their concentration each of the above listed can influence the yield and the efficiency of the extraction. The reduction with sodium borohydride promotes the conversion of free fructosyl-lysine into hexitol-lysine in order to avoid the overestimation of CML, CEL [120]. Moreover, the use of sodium borohydride is recommended when the concentration of free unstable Amadori products is high. Unfortunately, the use of this reducing agent had several drawbacks: protein degradation and free counterpart losses during the reduction, precipitation and purification procedure; moreover, the use of sodium borohydride can interfere with the release of furosine with the above mentioned reduction of fructosyl-lysine into hexitol-lysine. After several preliminary measurements it was decided to avoid the reduction. A good compromise between the detection of furosine and that of CML/CEL was achieved controlling the oxidation under nitrogen. In particular, prior the acidic hydrolysis the screw capped flasks were saturated with nitrogen in order to reduce the effect of autoxidation and control the reaction pathway [13].

The use of hydrochloric acid is a mandatory step for the hydrolysis of peptide bonds and for the release of amino acids, MRPs and for the conversion of fructosyl-lysine into furosine. Different concentrations of protein per mL of hydrochloric acid can lead to different efficiency of the hydrolysis with the consequent underestimation of lysine content. In the present study, the

extraction procedure was optimized in order to promote the dehydration reaction that leads to the formation of furosine and to the release of MRPs [69, 101]. Further studies will be conducted in order to compare the effect of time and concentration of hydrochloric acid on lysine release, mainly in protein rich samples.

The above described analytical performances were tested in food and feed samples in order to verify the robustness of the method. Several thermally treated foods were tested: powdered infant formula, low lactose milk, lab scale UHT milk, biscuits samples, bread (all prepared according to three different procedures previously described by our group) and powdered soybean-based feed products (prepared at industry scale). All data are summarized in **Table 3.3**. The concentration of CML in powdered infant formula analyzed ranged from 8.22 ± 0.31 mg/100 g of protein to 14.81 ± 0.92 mg/100 g of protein, while CEL and furosine ranged from 0.71 ± 0.02 mg/100 g of protein to 1.31 ± 0.11 mg/100 g of protein and 471.9± 22.3 mg/100 g of protein to 639.4± 21.1 mg/100 g of protein, respectively. The concentration of total lysine varied from 9.89 ± 0.88 to 13.12 ± 0.78 % of total protein. In low lactose milk the content of lysine was 5.21± 0.30 g/100 g of protein, while the concentration of CEL and furosine was 0.28 mg ± 0.01 mg/100 g of protein and 12.32 ± 0.31 mg/100 g of protein, respectively. CML was 1.28 mg ± 0.11 mg/100 g of protein and this value was perfectly in line with the one previously obtained. Lab scale UHT milk was prepared in order to verify the effect on raw cow milk; while the lysine content was of the same order of magnitude of the low lactose milk (4.71 ± 0.22 mg/100 g of protein), the concentration of the three markers of the MR was 18.41 ± 0.93, 1.12 ± 0.02 and 14.41 ± 1.02 mg/100 g of protein for CML, CEL and furosine respectively. The results obtained were perfectly in line with those previously obtained for the three categories of milk [109, 118], specifically the CML in low lactose milk was similar to one previously obtained by our group for LC-MS/MS analysis [9]. The concentration of CML and furosine was closed to the range previously obtained: 2.2 – 30.8 and 0.8 – 3.7 mg/100 g of protein for furosine and CML, respectively [121] .

In bakery products CML content was 43.75 ± 2.02 and 27.15 ± 0.61 mg/100 g of protein for biscuits samples and bread slices, respectively, while CEL and furosine were 46.25 ± 3.01 and 10.01 ± 0.61 and 10.91 ± 0.01 and 98.55 ± 4.61 mg/100 g of protein for biscuits and bread, respectively. The lysine content was almost similar in the two products: 5.01 ± 0.04 and 5.81 ± 0.04 g/100 g of protein, even if the protein content was 6% and 8% for biscuits and bread. The results here reported were of the same order of magnitude as the ones previously reported. Hull et al., analyzed several kinds of bread and other bakery products and the concentration of CML ranged from 2.6 to 45.1

mg/100 g of protein for wheaten bread and potato bread, respectively [122]. On the other hand He and coworker reported higher values for whole meal bread: CML ranged from 66.72 to 109.9 mg/100g of protein and CEL ranged from 53.30 to 82.04 mg/100 g protein for bread, while in biscuits samples the concentrations varied from 50.8 to 116.7 and 15.87 to 45.26 mg/100g protein for CML and CEL, respectively [123]. Interestingly, the concentration of furosine in bread (after 20 min at 200° C) is similar to the one reported by Capuano and coworker: after 13 min the concentration of furosine increased up to 200 mg/100 g of protein and it quickly decreased up to 20 mg/100 g protein at the end of the thermal treatment [124]. A similar kinetic profile was observed also by Ramirez-Jimenez and coworker in sliced bread: the concentration of furosine at the end of the process was 79.3 mg/100 g of protein while after 12 min it reached a concentration higher than 200 mg/100 g protein [125]. In biscuit samples the kinetic profile revealed similar trends to the ones obtained for bread; as a consequence at the end of the thermal process the concentration of furosine value of 10.01 ± 0.61 mg/100 g of protein was comparable to those of sucrose-containing cookies reported by previous authors [126].

Table 3.3: MRPs concentration after 8 replicates in different samples, the results for CML, CEL and furosine were reported as mg/100 g of protein, except for lysine. The results were compared to the AGE Database [127].

Food	CML	CEL	Furosine	Lysine (g/100 g protein)
Infant formula -1	8.22 ± 0.31	0.71 ± 0.02	471.91 ± 22.31	9.89 ± 0.88
Infant formula -2	10.4 ± 0.52	0.85 ± 0.06	542.53 ± 11.91	12.24 ±0.91
Infant formula -3	10.9 ± 1.03	1.10 ± 0.05	574.5 ± 44.12	13.12 ± 0.78
Infant formula -4	14.81 ± 0.92	1.31 ± 0.11	639.4 ± 21.11	10.28 ± 1.01
Age Database	0.6 – 40.5	/	Up to 1819	/
Low lactose milk	1.28 ± 0.11	0.28 ± 0.01	12.32 ± 0.31	5.21 ± 0.30
Age Database	1.4	/	/	/
Lab scale UHT milk	18.41 ± 0.93	1.12 ± 0.02	14.41 ±1.02	4.71 ± 0.22
Age Database	0.9-8.3	/	12.4 – 220.0	/
Biscuits	43.75 ± 2.02	46.25 ± 3.01	10.01 ± 0.61	5.01 ± 0.04
Bread slices	27.15 ± 0.61	10.91 ± 0.01	98.55 ± 4.61	5.81 ± 0.04
Age Database	2.6 – 45.1	/	/	/

The above described analytical performances were evaluated in industrially prepared soybean feeds in order to verify the main advantages of the method on industrial sampling. The simultaneous quantification of the four analytes allowed a direct overview of the extent of the MR, where the concentration of lysine and the formation of furosine, CEL and CML can be easily related to the final quality of foods using a single extraction and a single injection. According to the procedure described in material and methods section, soybeans were incubated at 110° C for one hour in an autoclave and the kinetic profile was reported in **Figure 3.3.** The initial concentration of lysine was

3.45 ± 0.12 g/100 g of protein while CML, CEL and furosine were 9.94 ± 0.74, 0.98 ± 0.04 and 24.24 ± 1.74 mg/100 g of protein respectively. After 30 minutes the concentration of furosine reached the highest values: 108.01 ± 8.97, then it rapidly decreased up to 60.58 ± 3.75 mg/100 g of protein after 55 min. According to the reaction mechanism the degradation of the Amadori products was followed by the increase of CML: at the end of the thermal treatment its concentration was higher than 76 mg/100 g of protein. CEL reached the maximum concentration after 45 minutes (2.41 ± 0.24 mg/100 g of protein), then it decreased probably due to degradation processes or to the blockage of methylglyoxal by other compounds. The degradation of lysine was constant throughout the thermal treatment, after 60 min lysine concentration was 2.60 ± 0.08 g/100 g of protein thus around 23%. Several studies reported the effect of soy proteins in the development of the MR focusing on soy health benefits and on the presence of functional molecules able to control the extent of the MR [128].

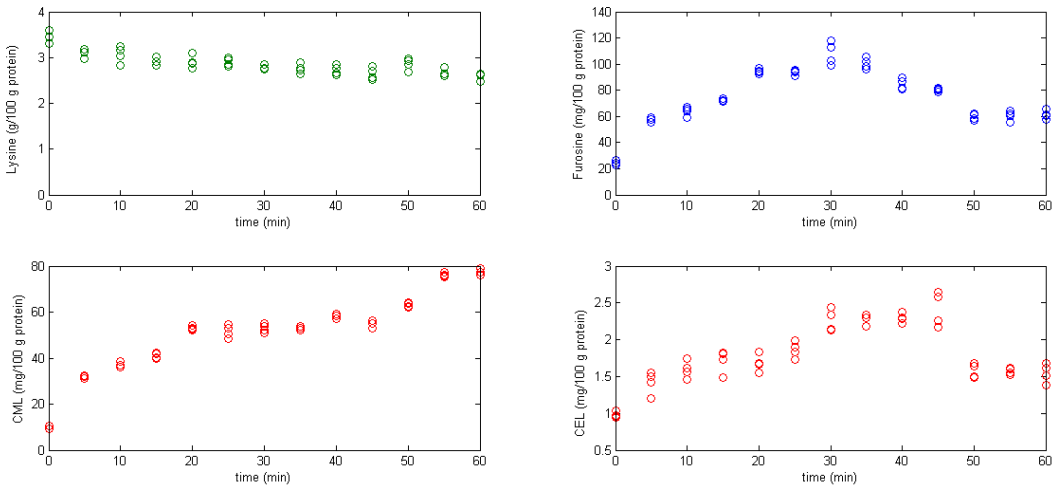

Figure 3.3: Kinetic profile of the precursor lysine (green), intermediate, furosine (blue) and end-products, CML and CEL (red).

This paper represents the first example of a systematic study on the relationship between thermal treatments, MR and soybean products in feeds and in pet food a topic recently attracting the attention of the scientific community. In fact, it has been observed that the average daily intake (mg/kg body weight$^{0.75}$) of HMF is 122 times higher for dogs and 38 times higher for cats than average intake for adult humans. Possible health risks, such diabetes and renal failure, can be associated to the intake of MRPs not only in human, but also in pets [129].

The analytical method allowed a comprehensive approach in the analysis of MRPs, simultaneously determining both lysine and its heat-induced derivatives. Up to now the golden standards for MRPs detection were RP-HPLC with UVvis detection for furosine and LC-MS/MS for CML, CEL and lysine, respectively. These results showed that the extraction procedure with nitrogen and hydrochloric acid provided a good compromise for the simultaneous detection of the four analytes. The analytical performances showed high sensitivity and good reproducibility and repeatability in several foods. Quantitative data were fully in line with those previously obtained by other authors on similar foods. The simultaneous detection of the four analytes offered a sensitive tool for the kinetic modeling on neoformed contaminant reaction routes monitoring the precursor lysine, the intermediate furosine via the indirect analysis of the Amadori products and the end-products CEL and CML. The simultaneous monitoring of all compounds allowed to minimize the variability among different samples and to combine the reaction steps starting from lysine blockage, Amadori compounds formation and fragmentation, CML and CEL formation.

Chapter 4

Quantitation of acrylamide in foods by High Resolution Mass Spectrometry

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Abstract

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

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

4.1 Introduction

Acrylamide has been a hot topic in Food Science and in particular in Maillard Chemistry,[130, 131] since the first report highlighted its presence at high concentration in French fries, cereals and coffee.[22] On one hand, overlooking acrylamide toxicological and exposure outcomes,[132-134] a huge variety of papers dealt with the chemical and analytical insights of acrylamide determination. The chemical aspects of acrylamide formation have been deepened in several pivotal papers: the determination of precursors,[135-137] the detection of a key intermediate,[138] the mechanism in heated food,[139] the kinetic modeling of formation.[140] On the other hand, in the last decade several methods for its quantitative determination have been reviewed.[21, 141] Generally, acrylamide analysis can be characterized by three steps: sample preparation including extraction, spiking with labeled internal standard with derivatization or not,[25] clean up, chromatographic separation and detection. As regards the first step several techniques have been developed providing satisfactory results: pressurized liquid extraction (PLE) cation-exchange cartridges, hydrophilic-lipophilic balanced cartridges, C-18 cartridges.[23, 142-144] The chromatographic separations should take into consideration several drawbacks, among them the acrylamide high polarity along with the poor retention, the matrix effect and the solvent interferences,[24, 145, 146] that can be ruled out through derivatization procedures with mercaptobenzoic acid or through the use of the "bromination" method.[147-149] For the detection step the analytical techniques of choice were the tandem mass spectrometry (MS/MS) acquisition performed by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) choosing the characteristic transitions *m/z* 72→55, 72→54 and 72→27,[26, 150, 151] Fourier transform infrared (FT-IR)[136] analysis or by time of flight mass spectrometry (TOF/MS),[28] either or by fluorescence method.[29] In order to obviate the matrix effect and the poor retention of acrylamide and in addition to the above mentioned analytical methods, many official institutions standardized and published their full validated methods for the determination of acrylamide, most of which focused on LC-MS/MS analysis and the isotope dilution with ^{13}C or ^{2}H (Swiss Federal Office of Public Health; US Food and Drug Administration).[26]

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

4.2 Materials and methods

4.2.1 Chemicals

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

4.2.2 High Resolution Mass Spectrometry (HRMS)

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

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

spray voltage 4.5 kV, the capillary voltage 42.5 V, the skimmer voltage 14 V, the capillary temperature 300 °C, the heater temperature 250 °C and a sheath gas flow 40 and auxiliary gas flow 6 (arbitrary units).

Before starting the acrylamide determination the instrument was externally calibrated by infusion with a calibration solution that consisted in caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v). Several reference masses (lock masses) were tested for the mass analyzer recalibration: methanol ($[M_2+H]^+$, exact mass: 65.05971); methanol/water ($[A_2B_2+H]^+$, exact mass: 101.08084); sodium adduct of methanol/water ($[A_2B_2+Na]^+$, exact mass: 123.06278); [2,3,3-d_3]-acrylamide ($[M+H]^+$,exact mass: 75.06322) and diisooctyl phthalate ($[M+H]^+$, exact mass: 391.28429).[152] In order to optimize the HRMS conditions and the mass accuracy, 200 µL of the above mentioned calibration solution were spiked with 10 µg of [2,3,3-d_3]-acrylamide and the scanning window was set in the range 70 – 1400 *m/z* only for the initial calibration procedure.

4.2.3 Preparation of standard solutions

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

4.2.4 Sample preparation

Cookies. Model cookies (height 3 mm diameter 30 mm) were prepared according to AACC method 10-54 (AACC, 2000) as previously described.[112] Cookies were baked at 200 °C for 13 min in a forced-air circulation oven (Memmert, Schwabach, Germany).

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

Brewed coffee and ground coffee. Ground coffee was purchased in a local market; an aliquot was directly analyzed without further preparation, instead 15 g were brewed with 100 mL of water (100 °C for 15 min) under continuous stirring. Solid particles were removed through a paper filter and 50 ml were stored at -20 °C until the analysis.

4.2.5 Acrylamide extraction

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

4.2.6 Tandem mass spectrometry (MS/MS)

In order to validate the HRMS method all the above mentioned samples were analyzed through LC/MS/MS according to Gökmen et al.[23] Two grams of the freeze dried French fries, cookies or coffee powder were weighed; 8.9 mL of deionized water was added in a 15 mL centrifuge tube along with 500 µL of Carrez reagent potassium salt, 500 µL of Carrez reagent zinc salt and 100 µL of internal standard [2,3,3-*d3*]-acrylamide (final concentration 90 ng/mL). For brewed coffee one mL was mixed along with 8.9 mL of water, 500 µL of both Carrez reagents and 100 µL of internal standard. The tubes were shaken vigorously for 1 min and the resulting mixture was centrifuged at 2500 x g for 10 min at 4 °C. Then supernatant was collected in a 20 mL volumetric flask, and two further extractions were performed using 5 mL of deionized water, except for brewed coffee that was filtered and injected. Finally, the pellets were discarded, and the supernatants were filtered through a 0.45 µm nylon filter. One milliliter of supernatants was collected and passed through an Oasis HLB cartridge (Waters, Milford, MA) previously activated with 1 mL of methanol and 1 mL of deionized water; 20 µL of the final solution was injected onto the LC column for quantitation by

MS/MS. Identification and quantitative determination of acrylamide and [2,3,3-*d3*]-acrylamide were carried out using an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, Carlsbad, CA) coupled to an ion spray interface, equipped with an HPLC binary micropump series 200 (Perkin-Elmer, Waltham MA). Chromatographic separation of acrylamide and [2,3,3-*d3*]-acrylamide was achieved through an Inertsil column, 250 × 4.6 mm, 5 μm (GLSciences, Torrance, CA), the mobile phases were A: 0.1% formic acid and B: 0.1% formic acid in methanol and the following gradient elution (min)/(% B): (0/0), (3/0), (8/93), (12/0), (15/0) was applied at a flow rate of 0.8 mL/min, whereof 0.2 mL were split into the ion source. The quantitation was carried out in multiple reaction monitoring (MRM) at *m/z* ratios of 72 and 75 for acrylamide and [2,3,3-*d3*]-acrylamide, respectively. Specific molecular fragments corresponding to *m/z* 55 and 44 for acrylamide and *m/z* 58 and 44 for [2,3,3-*d3*]-acrylamide were also monitored. The daughter ions were obtained through fragmentation with the following conditions: the source temperature was set at 350 °C, nitrogen was used as nebulizer gas at a flow rate of 12 L/h, and the needle and cone voltages were set at 3.0 kV and 100 V, respectively. Under the above-mentioned chromatographic conditions the acrylamide and its corresponding deuterated standard eluted at 7.4 min. For LC/MS/MS analysis acrylamide was quantified using a linear calibration curve built in the range 50−500 ng/mL, the LOD and LOQ were 10 and 20 ng/mL for acrylamide and [2,3,3-*d3*]-acrylamide, respectively and the coefficient of determination r^2 was higher than 0.99.

4.2.7 Statistical analysis

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

4.3 Results and discussion

4.3.1 Method development

Although many advances have been performed in acrylamide analysis, improvements in high-throughput quantitative procedures for its determination are still useful. The key point of this paper was to evaluate the potentialities of HRMS in the detection of this small amide in a complex

mixture riding out the problems associated to the solid phase extraction and, possibly, to the use of internal standard.[21, 141]

The first step of the work dealt with the optimization of chromatographic separation of acrylamide in combination with HRMS detection. It is well known that acrylamide is a very polar molecule with poor retention ($k' < 2.0$) in conventional liquid chromatography reversed phase sorbents.[23, 153] Four columns with different selectivity were tested evaluating chromatographic performances, including retention time and reproducibility. Among the tested columns, including HILIC phase, pentafluorophenyl phase, and a core shell C18 phase, a polar endcapped stationary phase was selected in order to get a good reproducibility and avoid the interferences due to the solvent, to the matrix and to the unretained compounds that eluted along with the solvent front. A typical chromatogram of acrylamide standard is presented in **Figure 4.1**. Along with polar endcapped column, the use of a core shell C18 phase can ensure a significant reproducibility for several subsequent injections even if the k' was always 1. From the chromatographic separation point of view, relevant results were obtained with HILIC mode, even if the use of acetonitrile interfered with the ionization of acrylamide.

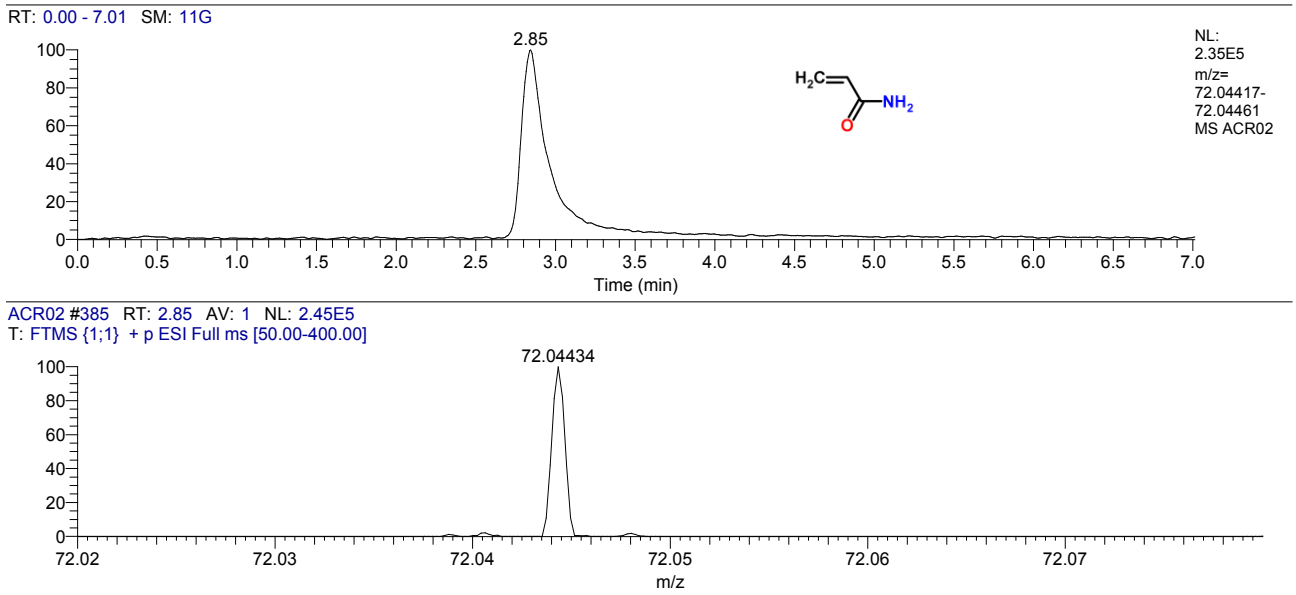

Figure 4.1 Typical extracted ions chromatogram and mass spectrum of acrylamide standard dissolved in water. Δ ppm = -0.7. Acrylamide theoretical mass: 72.04439**.**

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

The following step of the experimental work was the HRMS optimization downstream the U-HPLC separation. Specifically, the use of auxiliary gas coupled to a capillary voltage higher than 4.0 kV caused the fragmentation of acrylamide in source. As a consequence it was decided to use an auxiliary gas flow rate of 6 AU in order to avoid undesired fragmentation of the molecules and the formation of the typical fragment (72→55).[26, 150, 151, 154] The lens voltage was optimized setting the mass on 72.0 by manual tuning. This procedure was repeated in two different conditions: direct infusion of the acrylamide standard solution in the ion source and its infusion in the liquid chromatography flow in order to evaluate the interferences of the solvents. The heather temperature and the capillary temperature were optimized to ensure the best solvent nebulization. Under the conditions stated in the method session, Orbitrap MS analyzer showed the best compromise between selectivity and quantitative performance.

Table 4.1: Contaminant or interference ions used as locking mass to recalibrate the instruments during the run [152].

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

4.3.2 Method performance

The method performance was tested against quality parameters such as specificity, carryover, linearity of the calibration, coefficient of correlation (r^2), limit of quantitation, limit of detection, precision, accuracy and recovery. The first point was checking for the absence of any contaminants with an exact mass similar to the one of acrylamide injecting several times pure water. Subsequently a calibration curve was built after determining the LOD and the LOQ. The LOD was calculated according to Armbruster et al. [115] After twenty replicates the limit of blank was 0.5 ppb, the limit of detection was 2.65 ppb and the limit of quantitation was 5 ppb. Two different series of calibration curves were prepared: one spiked with the deuterated internal standard and another one without internal standard. For both series the linearity was achieved in the range 5-500 ng/mL and the r^2 was always higher than 0.99. Each point was injected three time each day and for

three consecutive days. After each replicates a blank sample (pure water) was injected in order to verify the absence of carry-over effect. The precision of the two methods was verified in the linearity range through the evaluation of the slope of three different calibration curves; the results, reported as % RSD, were lower than 8% for both calibration curves; the accuracy of the method, calculated through the ratio observed amount/specified amount x 100, was always higher than 90%. The recovery test was performed using two different concentration of [2,3,3-d_3]-acrylamide, (100 ng/mL and 253 ng/mL final concentration, n=6 for each matrix) in order to verify the matrix effect and the ion suppression. First the ionization performance of [2,3,3-d_3]-acrylamide was evaluated through the injection of the pure standard dissolved in water and then the recovery was calculated as the ratio between area internal standard and the area of spiked samples. The results ranged from 90% to 99% and they are shown in **Table 4.2**. In **Figure 4.2** the recovery of internal standard spiked in French fries is presented. The set-up of the condition for mass accuracy optimization was one of the prominent point of the paper. In a first set of fine tuning trials several lock masses were used to recalibrate the instrument and reduce the mass error[152] and the mass to charge ratio of some contaminants or interference ions was evaluated (**Table 4.1**), such as methanol ($[M_2+H]^+$,exact mass: 65.05971); methanol/water ($[A_2B_2+H]^+$, exact mass: 101.08084); sodium adduct of methanol/water ($[A_2B_2+Na]^+$, exact mass: 123.06278). Then, 1 µL of [2,3,3-d_3]-acrylamide solution ($[M+H]^+$,exact mass:75.06322) was directly infused in the liquid chromatography flow with a final concentration of 3 µg/mL. The former trials gave unsatisfying results with a mass accuracy around 5 ppm, while the latter trial, which used deuterated acrylamide straight in the calibration solution reduced the mass accuracy to -0.7 ppm. The calibration mixture was spiked with 10 µg of [2,3,3-d_3]-acrylamide in order to set the instrument for the optimal detection of small molecules with an *m/z* lower than 80. The presence of [2,3,3-d_3]-acrylamide in the calibration solution determined the reduction in the discrepancies between the observed mass ($[M+H]^+$,exact mass:72.04434) and the theoretical mass of acrylamide ($[M+H]^+$,exact mass:72.04439).

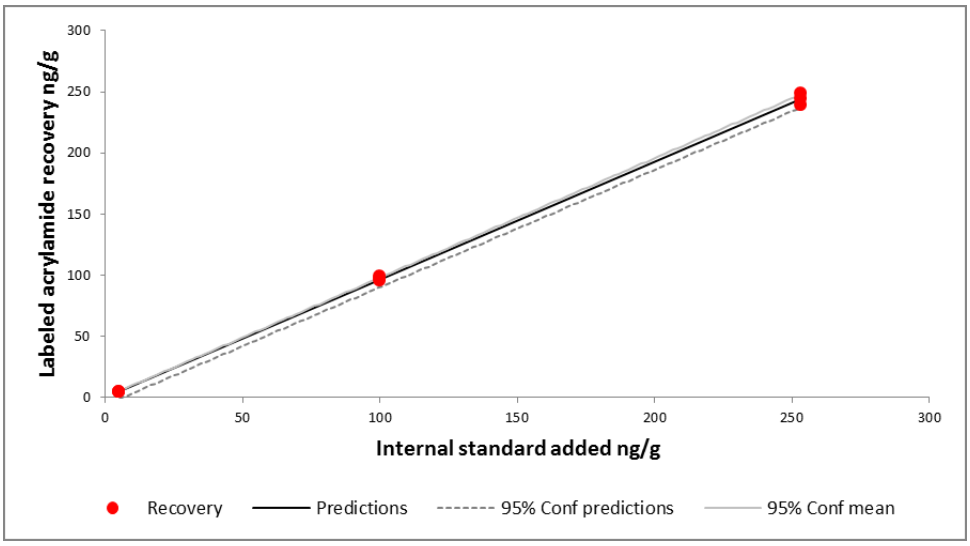

Figure 4.2: Recovery of internal standard spiked in French fries. The dotted lines represent the 95% confidence intervals calculated on the predictions and on the mean (n=3).

4.3.3 Measurement of acrylamide content in foods

As it is described above, three of the most commonly consumed acrylamide-containing food, such as French fries, coffee and cookies, were selected to test the performance of the developed method. The determination of acrylamide in these food matrices showed different requirements in terms of extraction and clean-up before chromatographic separation.[21] Up to now acrylamide was detected through solid phase extraction along with liquid chromatography and tandem mass spectrometry detection;[26, 154-157] by LC/MS after derivatization with 2-mercaptobenzoic acid,[25] by direct gas chromatography coupled to mass spectrometry[158] and by pyrolysis GC/MS.[159] The extraction procedure used in this paper was exactly the same for cookies, French fries and ground coffee while for brewed coffee only a dilution in water was performed. The recovery test and the LOD were evaluated in the different matrix, with satisfactory results as presented in **Table 4.2,** where the LOD and LOQ in the tested foods and in standard solutions were almost similar**.**

Table 4.2: LC/HRMS performances for acrylamide detection in three different foods. %RSD* indicates the set of samples performed with labeled internal standard. LOD and LOQ (ppb).

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

In **Table 4.3** acrylamide concentrations obtained on the various foods with and without the use of internal standard were reported and they were compared not only with those present in the literature, but also with the results obtained by LC/MS/MS on the same samples. For French fries the value ranged from 270 ng/g ± 12.1 ng/g (254 ± 4.6 ng/g for internal standard analysis) to 424 ± 10.3 ng/g (490 ± 10.0 ng/g for internal standard analysis) according to the processing time (five and seven minutes respectively) [160]. These values were really similar to the ones obtained through LC/MS/MS as reported in **Table 4.3** [17, 151, 161]. For cookies samples two different typologies of cookies prepared *de novo* according to two recipes, already used in a previous paper from our group, were used for acrylamide determination.[112] The value varied from 276 ± 3.5 ng/g (cookies with 0.65% sodium chloride) to 317 ± 9.2 ng/g (cookies without 0.65% sodium chloride). The use of the internal standard resulted in similar values: 262 ± 4.0 ng/g and 340 ± 5.6 ng/g for cookies with sodium chloride and without, respectively. These data clearly showed the substantial agreement between the U-HPLC/HRMS method and the SPE LC/MS/MS method **(Table 4.3)**.

The method performances were tested also in brewed coffee sample and in ground coffee. In this case the acrylamide content was 12.1 ± 0.1 ng/mL and 140 ± 3.6 ng/g for brewed coffee and ground coffee, respectively and the previous results were fully confirmed,[162-165] even if with the use of [2,3,3-d_3]-acrylamide small differences were present: 15 ± 0.2 ng/mL and 159 ± 8.1 ng/g for brewed coffee and ground coffee, respectively. The key points for brewed coffee were the LOD and the recovery that were 3 ppb and 90%, respectively. Acrylamide detection in coffee is characterized by several drawbacks due to the matrix effect and to the roasting of coffee beans.[162] For this

reason for LC/MS/MS the use of internal standard is a key point that can be easily overcome thanks to the HRMS. This was presented in **Table 4.3** where the analysis performed through HRMS or MS/MS showed similar results.

Table 4.3: Acrylamide concentration in the tested foods, through LC/HRMS (with and without labeled internal standard); through LC/MS/MS and literature data.

Food	No labeled standard	Labeled standard	LC/MS/MS	Literature data	Ref.
Cookies (different formulations)	276 ± 3.5 ng/g	262 ± 4.0 ng/g	286 ± 15.3 ng/g	278-313 ng/g	[112]
	317 ± 9.2 ng/g	340 ± 5.6 ng/g	325 ± 9.3 ng/g	451-510	[25]
French fries (different frying time)	270 ± 12.1 ng/g	254 ± 4.6 ng/g	280 ± 18.8 ng/g	253-2688	[17]
	424. ± 10.3 ng/g	490 ± 10.0 ng/g	409 ± 11.1 ng/g	150-1200	[161]
				50-1800	[151]
Brewed coffee	12 ± 0.1 ng/mL	15 ± 0.2 ng/mL	13 ± 0.2 ng/mL	6-16 ng/mL	[164]
				5-12	[162]
				14-21	[163, 166]
Ground coffee	140 ± 3.6 ng/g	159 ± 8.1 ng/g	145 ± 3.2ng/g	45 – 374 ng/g	[164]
				267; RSD %: 0.5	[165]

The results obtained with the developed methods were in any case well in line with those present in literature.[166, 167] From the analytical point of view the use of [2,3,3-d_3]-acrylamide increased the robustness of the method, in particular the variability due to the ion suppression or to the matrix effect were eliminated for each analysis. Interestingly without the use of labeled internal standard the results were still satisfying and the differences between the two methods were less than 14% in solid foods, thus suggesting that in the cases not requiring a high precision the use of HRMS method could theoretically allow also to avoid the use of external standard.

A huge variety of papers has been published on quantitation procedure for acrylamide in foods. The method here developed using U-HPLC Orbitrap HRMS proved to be very effective as it allowed to ride out the matrix effect and the time consumption due to solid phase extraction. Moreover the use

of a modified calibration solution allowed the optimal set of the instrument and it improved enormously the mass accuracy for acrylamide detection. Also the use of internal standard could be theoretically avoided with a negligible loss in method accuracy. From the analytical standpoint the performances were satisfactory: the relative standard deviation was less than 7%, the recovery in the three different matrix was always higher than 90% and the limit of detection was comparable to the ones of tandem mass spectrometry.

Chapter 5

Targeted metabolite profile of food bioactive compounds by Orbitrap High Resolution Mass Spectrometry: the "FancyTiles" approach

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Abstract

In this paper a new targeted metabolic profile approach using Orbitrap High Resolution Mass Spectrometry was described. For each food matrix various classes of bioactive compounds and some specific metabolites of interest were selected on the basis of the existing knowledge creating an easy-to-read fingerprinting named "FancyTiles". The procedure resulted in a plot of semi-quantitative data allowed to highlight for each food the main metabolites related to the biological or sensorial attributes within an educated schema. Results showed that the FancyTile procedure is an useful tool for research programs aiming at improving the health potential of food and ingredients.
In this paper the FancyTile was described and it was successfully applied to verify the differences in the metabolic profile. Olive oils from different cultivars, waste mill waters from olive grown in different location and artichokes cultivated with different agronomical practices was used as case study.

Keywords: Metabolic profile, High Resolution Mass Spectrometry, Orbitrap, "FancyTiles", Functional Foods

5.1 Introduction

Metabolomic is usually defined as the global quantitative assessment of metabolites in a biological system and it is emerging also in food science as a tool for assessing raw materials and processing quality [168]. Metabolite pattern is, in fact, the result of the interaction of the genome (i.e. vegetable cultivars) with its environment (i.e. vegetable cultivation location or agronomic techniques) and then with technological transformations. For these reasons metabolomics is often considered a powerful tool to provide an instantaneous snapshot of food micro components [169].

Metabolomic analyses have been generally classified as targeted or untargeted. Targeted analyses focus on a specific and small group of intended metabolites with most cases requiring accurate quantification [170]. In contrast, untargeted or comprehensive metabolomics focuses on the detection of as many metabolites as possible in order to obtain patterns or fingerprints without focusing on specific compounds [171].

Several analytical instruments are currently used in food metabolomic analysis being NMR [172, 173]; and LC/MS [174, 175] , CE/MS [176], SFC/MS [177, 178]; and GC/MS [179] the most useful techniques. However, because metabolites vary greatly (e.g., in molecular weight, polarity, solubility, partition coefficient etc.), the simultaneous detection is really difficult and it is necessary to narrow the number of target metabolites and acquire data using instruments and/or combine data from several instruments, as appropriate [180].

Recently, metabolomic studies have been performed using Orbitrap High Resolution Mass Spectrometry (HRMS) [181-183]. Briefly, this mass analyzer is a non-conventional ion trap where the ions are trapped in an electrostatic field which forces ions to move in complex spiral patterns oscillating back and forth around the detector. The presence of an electrode allowed the detection of the axial component of these oscillations and a Fourier transform is employed to obtain oscillation frequencies for the ions with different masses, resulting in a very accurate reading of their *m/z* [31]. As a consequence the Orbitrap mass analyzer can detect ions using high-resolution accurate mass. This point represents one of the most prominent advantage of the Orbitrap whose resolving power is around 100000 according to the analysis. A full scan method can be used, looking for each ion in the appropriate scan range; as a consequence it is possible to detect a wide number of compounds and the same analysis can be used to study a complete metabolic pattern of different classes of metabolites. Furthermore, the retrospective “post-targeted” evaluation of old data for the detection of non-“a priori” selected compounds by reconstructing any desired reconstructed ion chromatogram is an advantage toward the targeted MRM approach [184]. Thanks to the mass

spectrometry features, the sample preparation becomes as simple as possible in order to detect a wide range of molecules and as a consequences the chromatographic runs are short and characterized by a rapid linear elution gradient.
A new metabolomic approach using Orbitrap HRMS was described in this paper: it involved a targeted metabolite profiling approach including a wide range of metabolites from different classes. For each food matrix the classes of compounds and the specific metabolites were selected on the basis of the existing knowledge. The objective is to create for each specific food an easy-to-read fingerprinting highlighting the main metabolites related to the biological or sensorial attributes within an educated schema.
The main goals were: (1) to develop a rapid, simple and general method for the simultaneous detection and identification of a wide range of metabolites in food, (2) to create an original data representation output providing an immediate representation of the variation among different samples of the same food.
The new representation method was named "FancyTiles" and some case study were described in this paper as proof of concept: the comparison of metabolite profile allowed to evaluate genetic, environmental or technological effects within the considered samplings.

5.2 Material and methods

5.2.1 Chemicals

Acetonitrile and water were for LC/HRMS determination were obtained from Merck (Darmstadt, Germany); formic acid and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). The calibration solutions (see in HRMS analysis section) were obtained from Thermo Fisher Scientific (Bremen, Germany).

5.2.2 Sampling

Phenolic extracts from extra virgin olive oils from two different olive cultivars (*Corotina* and *Bosana*) were extracted according to Montedoro et al. [185]. Olive waste mill water (OWMW) was experimentally obtained in laboratory scale in order to simulate oil extraction under controlled and constant conditions. Olive cv. *Ogliarola* were obtained from two different field located in Puglia, South Italy, near Bari and near Brindisi respectively; olive were pressed and, after centrifugation, oil and OWMW were separated. OWMW samples were diluted in water 1:10, centrifuged at 2800g

for 10 min at room temperature and filtered through a 0.45 μm Whatman filter paper (Whatman International Ltd., Maidstone, U.K.).

Artichokes *'Romanesco type' cv C3* were grown in experimental field and mycorrhized plants were inoculated at transplanting with beneficial bacteria. After harvesting, edible part was separated and a simple clean-up was performed by using methanol/water (70:30, v/v) and sonicated at room temperature for 30 min. The mixtures were centrifuged at 2800g for 10 min at room temperature, filtered through a 0.45 μm Whatman filter paper (Whatman International Ltd., Maidstone, U.K.)

5.2.3 High resolution mass spectrometry (HRMS) analysis

LC-MS data were acquired on an Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (10° C) and a column oven. Chromatographic separation was carried out on a Gemini C18-110Å 5 μm column (150 mm x 2.0 mm) (Phenomenex, Torrance, CA) thermostated at 30°C. For secoiridoids and anthocyanin analysis the mobile phases consisted in 0.3% TFA in water (solvent A) and 0.3% TFA in acetonitrile (solvent B). Gradient elution was linearly programmed as follows: 5% B (1 min), 5-15% B (1 min), constant to 15% B (2 min), 15- 100% B (4 min), constant to 100% B (2 min), 100-5% B (2 min). For all the other phenolic compounds and guaianolides analysis, 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used. Gradient elution was linearly programmed as follows: 10% B (1 min), 10-90% B (7) min, constant to 90% B (2 min), 90-10% B (2 min). The flow rate was set to 200 μL/min and the injection volume was 10 μL. The U-HPLC was directly interfaced to an Exactive Orbitrap MS equipped with a heated electrospray interface (HESI). Mass spectrometer operated in the full spectral acquisition mode, where the positive and negative ionization mode was used in the same run in the mass range of *m/z* 65–1300. The resolving power was set to 50000 full width at half-maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s. The automatic gain control was used to fill the C-trap and gain accuracy in mass measurements (ultimate mass accuracy mode, $5x10^5$ ions); maximum injection time was 100 ms. The interface parameters were as follows: the spray voltage was +3.5 kV and -3.0 kV in positive and negative ion mode, respectively; the tube lens was at 100V (-100 V in negative ion), the capillary voltage was 30 V (-50 V in negative ion), the capillary temperature was 275 °C, and a sheath and auxiliary gas flow of 30 and 15 arbitrary units were used.

Before starting the HRMS analysis the instrument was externally calibrated by infusion with two different calibration solutions: for positive ion calibration mode, the solution consisted in caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v); instead the negative ion mode calibration solution consisted in sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 in acetonitrile/methanol/water solution (2:1:1, v/v/v) containing 1% acetic acid. The exact mass of diisooctyl phthalate ($[M+H]^+$: 391.28429) was used as lock mass for internal standard recalibration.

5.2.4 Data analysis

Metabolite identification was performed by using exact mass values value up to the fifth decimal digit with mass tolerance ± 5 ppm and in the Supplementary Contents section a table with retention time, theoretical mass, experimental mass and error was reported. Three different injections for each samples were performed interday and intraday and only analytes showing high reproducibility (interday and intraday area variability <0.5%) were taken into account. Chromatographic data acquisition and peak integration were performed using Xcalibur software (Thermo Fisher Scientific, San Jose, USA), with a specifically modified Excel macro. Data were processed using XLStat 3D-Pro statistical software (Addinsoft, New York, NY). For each analyzed product, a specific scheme was designed including the main phytochemicals, free amino acid and glycated amino acids on the basis of literature data and grouping family of compounds in the same column. Then peak area values were converted into logarithmic scale and, automatically, a FancyTile for each sample was built by plotting peak area values on a logarithmic colorimetric scale from blue (minimum value) to red (maximum value).

5.3 Results and discussion

5.3.1 Construction of a "FancyTiles" flow chart

The flow chart in **Figure 5.1** highlighted the steps leading from the metabolic profile analysis to the construction of a FancyTile. The first step was the crucial point of the work: for each food or ingredient an accurate literature study allowed to perform an educated guess in order to select the main classes of bioactive compounds having potential biological activities that could be of relevance for human health or for sensory aspects. On this basis a schema in form of a FancyTile was created. Each metabolite is represented by a square and the set of all the squares represents the metabolic profile. In each FancyTile the different families of compounds were grouped in the same

column and within the same column metabolite were ordered on the basis of their relevance. Ideally, in the FancyTile for each food or ingredient it would be possible to have in a glance the pictures of all interesting compounds.

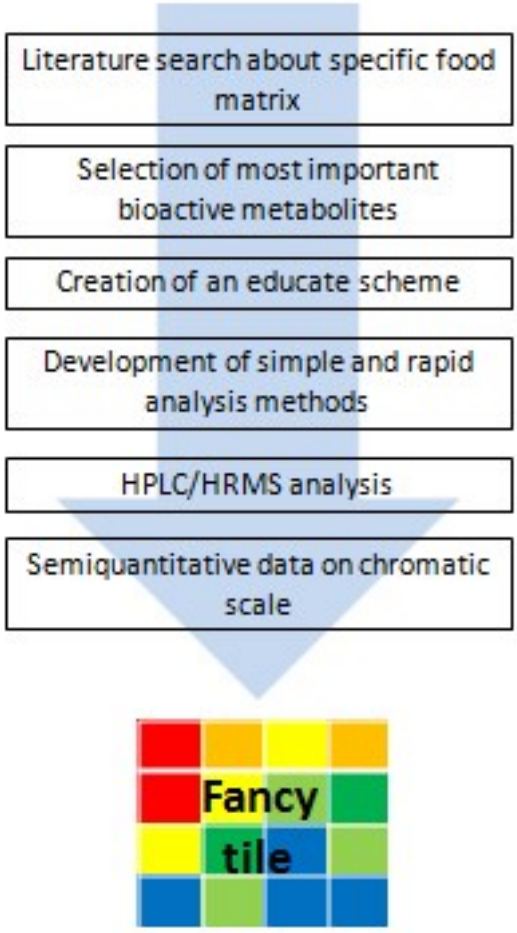

Figure 5.1: The "FancyTile approach" flow chart.

After identifying the classes of interest, a simple, rapid and inclusive analytical method was developed. According to the above mentioned approach, extraction step was as simple as possible or totally absent: in many cases food samples were only ground, filtered and diluted before injection. Any purification step was avoided thus reducing time of analysis and ensuring an high reproducibility. The other side of the coin was that the extraction, although reproducible, was not quantitative for all compounds present in the sample. Simple and rapid chromatographic conditions were then studied in order to analyze simultaneously several classes of compounds. This objective was not always possible: for secoiridoids and anthocyanins, it was necessary to modify chromatographic method, even if the same column and the short chromatographic approach was used. The contemporary data acquisition in positive and negative ion mode allowed the detection of

a huge variety of analytes within the same chromatographic run. Orbitrap mass analyzer guaranteed high interday and intraday signal reproducibility [186] so, it was possible to perform a semi-quantification procedure on MS peak area values. Therefore it should be very clear that the FancyTile procedure do not employ a calibration curves for each compounds (or classes of compounds) as the purpose of this approach was not a quantitative determination of each compounds but to get a metabolite profile allowing and a rapid comparison among set of similar samples differing for one or few variables.

Last step of the analysis was to develop a semi-automated method to elaborate data and give an immediate snapshot to appreciate the differences among samples. This was achieved transferring the intensity of the MS peaks into a diagram (named FancyTile) showing all relevant metabolites, arranged according a chromatic scale.

In **Figure 5.2** the construction of an hypothetical tomato FancyTile was shown. Tomato was chosen because it contained various classes of compounds of interest. The most important class of bioactive compounds are phenolics and carotenoids [187] so in a theoretical "tomato FancyTile" phenolic acids, flavonoids, anthocyanin and carotenoids should be reported,. *Solanaceae* plants (like tomato) are also well known for their content of glycoalkaloids [188] so this class of compounds could be included in a theoretical "tomato Fancy tile". Tomato is usually subjected to transformation and thermal treatments and, for this reason, it can be also interesting to evaluate in a "tomato Fancy tile" some markers of thermal stress such as Maillard reaction end-products (MRPs), free and glycated amino acids contents and their ratio [13, 189, 190].

It is clear that according to the different foods or ingredients the compounds characteristic of each product should be searched: for example glucosinolates in *Brassicaceae*, secoiridoids in oils, capsaicinoids in pepper or guaianolides in *Asteracee*, anthocianidins and tannins in wine offer a partial scenario of the possible characterization.

In the following paragraphs some applications of the FancyTile approach were reported to highlight the potentialities of this approach.

Phenolic acids	Flavonoids	Anthocyanin	Carotenoids	Glyco alkaloids	Free AA	Glycated AA	MRPs
Caffeic ac. hexoside	Rutin	Petunidin coumaroyl rutinoside	beta - carotene	Tomatin	Glu	Fru-Glu	Homoserine
Chloro genic ac. hexoside	Naringin	Malvidin coumaroyl rutinoside	Lycopene	alfa-tomatin	Asn	Fru-Asn	Dehydro alanine
Couma roylquinic ac.	Narin genin	Delphini din rutino side gluc	Phytoene	Deidro tomatin	Phe	Fru-Phe	Homo cysteine
Caffeic ac.	Naringe nin gluco side	Petunidin rutinoside glucoside	Lutein	Licope roside	Ser	Fru-Ser	Lys-Ala
Ferulic ac.	Kampferol rutinoside	...	Phyto fluene	Tomato side	Tyr	Fru-Tyr	Furosine
Protocate chuic ac.	Quercetin		...	Esculeo side	Ala	Fru-Ala	CML
...	...			...	...	...	HMF

Figure 5.2: Example of theoretical FancyTile: metabolite fingerprinting in tomato. AA: Amino acid; MRPs: Maillard Reaction end-products.

5.3.2 Extra virgin olive oil

Olive oil represented the most consumed dietary edible fat in Mediterranean basin and its consumption was correlated to the lower incidence of coronary heart disease and some cancers [191]. It was demonstrated that these effects should be also attributed also to the phenolic fraction of olive oil [192, 193] which are only present in the not-refined olive oil. EFSA recently released an health claim on the cardiovascular benefits related to the consumption of phenolic-rich, bitter olive oil [194].

Olive oil is a source of at least 30 phenolic compounds [195, 196]. The phenolic composition varies greatly in quantity and quality depending on age of the tree, agricultural techniques, degree of ripeness, soil composition, climate, processing technique and storage [197-200]. Among these, the cultivar has the most significant impact on the phenolic composition of virgin olive oil [201], and a statistical methods based on the metabolites concentration in monovarietal olive oils was explored to recognize their origin [202].

Olive oil phenolic composition was well investigated using long chromatographic runs in order to well separate each compounds and accurate extraction steps for quantitative determinations [203]. Recently, a new interesting approach using LC/HRMS was introduced for the quantitative and qualitative assessment of olive [204]. In this study, two rapid chromatographic runs allowed a complete characterization of phenolic olive oil fraction allowing to highlight the differences among two olive cultivars.

As shown in **Figure 5.3**, olive oil phenols can be divided into 5 different classes: namely secoiridoids, phenyl ethyl alcohols, lignans, flavonoids and phenolic acids. Secoiridoids are the most abundant phenolic compounds and the most typical ones because they are exclusive to plants belonging to the Family of *Olearaceae* that includes *Olea europaea L. Olive.* According to previous works [193], the most abundant secoiridoids are the oleuropein aglycone (DHPEA-EA) and the dialdehydic form of elenolic acid linked to hydroxyphenil-ethanol (HPEA-EA), originated during crushing by the hydrolysis of oleuropein. Oleuropein and derivatives exert anti-inflammatory effects, reducing the tissue damage in rats [205] and are able to act as Tau aggregation [206]. Both our samples showed very high DHPEA-EA concentrations, but *Coratina* oil was richer in secoiridoids than *Bosana* oil: also HPEA-EA and elenolic acid, in fact, was detected at the highest level in *Coratina* oil and HPEA-EDA (dialdehydic form of elenolic acid linked to DHPEA) was found in *Coratina* oil at higher level than in *Bosana* oil. On the other hand ligstroside, the glucoside of the tyrosol derived secoiridoids, was found at relevant concentration in *Bosana* oil, and it was not present in *Coratina* oil.

Coratina oil was richer than *Bosana* oil not only in secoiridoids fraction but also in flavonoid components. According to previous works [207] apigenin and quercetin aglicones were found in virgin olive oil but in *Coratina* oil we also found traces of apigenin glucoside and higher level of quercetin than in *Bosana* oil.

On the other hand, *Bosana* oil had an higher concentration of simple phenolic compounds, hydroxytyrosol in particular. Along with secoiridoids, this is one of most typical constituent in olive oil [199] and in comparison with other oil phenolics, it showed higher antioxidant activity [191].

Hydroxytyrosol was reported to reduce the risk of coronary heart disease and atherosclerosis [208], to inhibit arachidonic acid lipoxygenase [209] or to inhibit platelet aggregation [210].

The comparison of lignans between the analyzed oils was more complex: *Coratina* showed higher level of acetoxypinoresinol but in *Bosana* were found higher concentration of syringaresinol and little amount of pinoresinol. Also lignans greatly contribute to antioxidant activity and nutritional value of virgin olive oil: a correlation between the antioxidant activity of the phenolic extract and the lignans concentration was reported in a previous paper [209].

According to previous works [211-213] minor amount of phenolic acids were also found in virgin olive oil: in particular sinapic, caffeic, ferulic, syringic and vanillic acid were present in our samples with slight differences between two samples.

In conclusion, cultivar strongly influenced each phenolic class and, as previously reported [212], *Coratina* oil is richer in phenols than *Bosana* oil but specific differences were observed in the various class of compounds. The FancyTile approach provided an immediate visual comparison and characterization of all bioactive phenolic compounds in virgin olive oil.

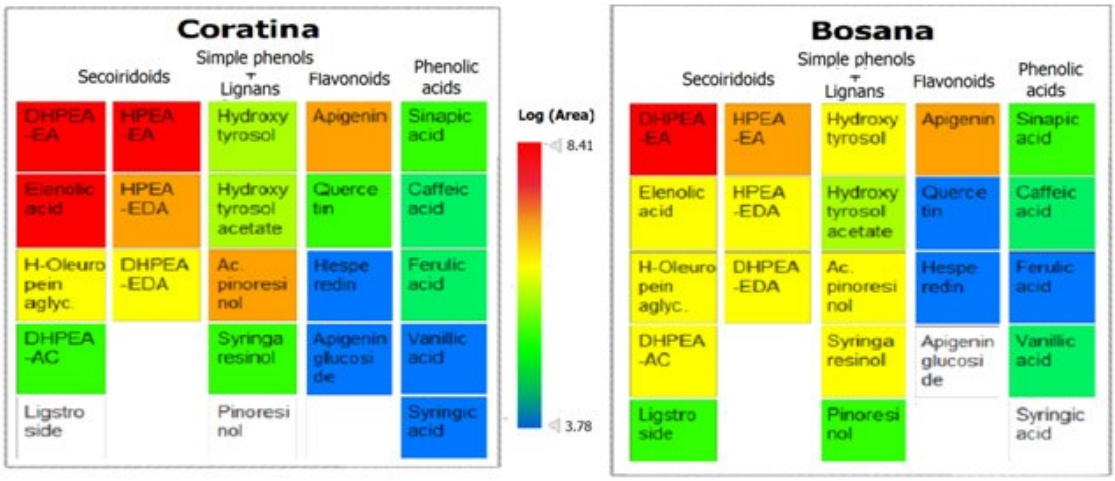

Figure 5.3: Example of FancyTile: phenolic metabolite comparison between extra virgin olive oil from two different cultivars.

5.3.3 Olive mill wastewater (OMWW)

In the olive oil production process, the disposal of olive oil mill waste waters (OMWW) represented an unsolved environmental problem but at the same time OMWW are rich in phenolic compounds and can be a source of bioactive molecules. Olives are rich in phenolic compounds, but only 2% of the total phenolic content of the olive fruit passes to the oil phase, while the remaining amount is lost in the OMWW [214]. This is due to the partition coefficients (oil/water) of most olive biophenols, ranging from $6x10^{-4}$ to 1.5 [215], which are in favor of the water phase. There is a

growing interest towards this olive oil by-products and several authors studied OMWW phenolic composition. In most cases, analysis comprises a step of solvent extraction and a long chromatographic run [216, 217]. Using the FancyTile approach a complete comparison of the phenolic compounds pattern of OMWW obtained by the same olive cultivar (Ogliarola variety) cultivated in two geographical region and milled in the same conditions was obtained (see **Figure 5.4)**. Samples from olive grown in Brindisi showed only traces of oleuropein, while in samples from olive grown in Bari slightly higher concentrations were detected but oleuropein aglycone (DHPEA-EA) and dialdehydic form of elenolic acid (DHPEA-EDA and HPEA-EDA) were not detected.

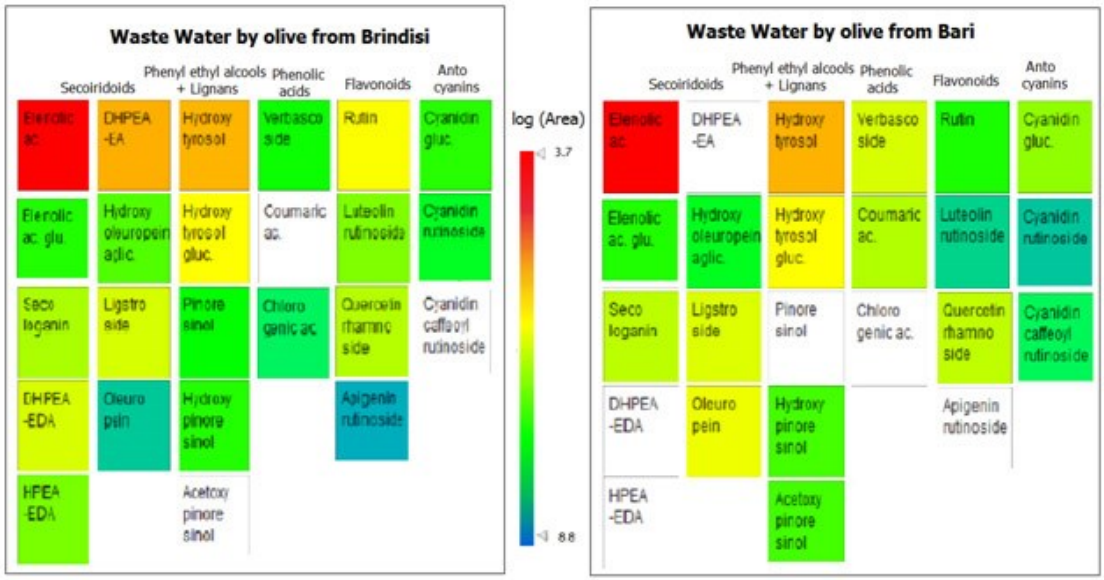

Figure 5.4: Example of FancyTile: phenolic metabolite comparison between extra virgin olive oil from two different cultivars by *Ogliarola* olive grown near Brindisi and near Bari, respectively.

Among secoiridoids derivatives hydroxytyrosol was one of the most abundant: it is claimed that hydroxytyrosol is formed in part as a result of hydrolysis of oleuropein (the major biophenol in many olive varieties) during oil extraction by the action of esterases [218]. No difference was found between the two analyzed samples in hydroxytyrosol and hydroxytyrosol glucosides content: they are important not only for their high concentration but also for their noteworthy antioxidant activities and potentially beneficial health properties [219, 220].

OMWW comprises also phenolic compounds with high molecular weight such as lignans [221] in particular pinoresinol and derivatives. In OMWW from Brindisi pinoresinol was detected while acetoxypinoresinol was not, conversely in OMWW from Bari acetoxypinoresinol was detected and

not pinoresinol, thus suggesting that lignans could be modulated through a specific interaction with the environment.

Other phenolic classes were less abundant in analyzed OMWW samples. Among phenolic acids and derivatives, the most typical compound is verbascoside, a complex polyphenol characterized by healthy beneficial properties [222]: it was detected in both OMWW, while coumaric acid was only present in samples from Bari and chlorogenic acid was present only in samples from Brindisi.

According to a previous work [223] flavonoids are present in OMWW in lower amount respect to other phenolic compounds and, looking at our samples, flavonoid fraction was less important in product from Bari than in product from Brindisi.

Cyanidin glucoside was the most abundant anthocyanin in both OMWW while cyanidin caffeoyl rutinoside was detected only in product from Bari. The anthocyanins presence (cyaniding derivatives, in particular) in olive fruits it was highlighted [193] and due to their polarity it is not surprising that they are typically present in OMWW [217].

In conclusion, data confirmed that location of olive growth greatly influenced presence of phenolic metabolite in OMWW: the developed OMWW FancyTile is a semi-automatic system allowing to evaluate quantitative and qualitative differences among samples.

5.3.4 Artichoke

In a previous study Palermo and coworkers analyzed artichoke phenolic fraction and showed a wide pattern of 24 phenolic acid and derivatives and 39 flavonoids in order to understand, from an agronomic point of view, plant response to external stresses [224].. In the framework of FancyTile development here a deep study of literature was performed to identify the most important marker-compounds of the artichoke and also the guaianolides compounds were included in the graphical presentation of artichoke data. Caffeic acid derivatives are the main phenolic compounds in artichoke, with a wide range of caffeoylquinic acid derivatives and chlorogenic acid, (5-O-caffeoylquinic acid) and di-caffeoylquinic acid (also known as cynarine) as the most important of these derivatives [225, 226]. Within phenolic acids, also ferulic acid and derivatives were reported in artichoke extracts [227, 228]. Other important phenolics such as the flavonoids have been tentative identified in artichoke tissues: in particular apigenin, luteolin, quercetin and naringenin were reported [229-231]. The functional properties of the edible flower of artichoke *Cynara scolymus L.* have been clearly linked to above described artichoke polyphenolic fraction [232-234].

Other characteristic bioactive compounds found in artichoke were sesquiterpene lactones (or guaianolides) such as grosheimin, cynaropicrin and cynaratriol [235]. They are the predominant bitter principles of artichoke and they also had biological and therapeutic activity including anti-inflammatory, antitumoral, antimicrobial, anthelmintic and anti-feeding [236]. Traditional method for sesquiterpene lactones analysis involved a long and complex extraction phase [237].The rapid approach used in this study allowed the identification of four guaianolides with the same simple clean-up procedure, the same chromatographic run and the same HRMS conditions used for phenolic compounds [238]. Three different chemical classes (phenolic acids, flavonoids and guaianolides) were reported in the same FancyTile (**Figure 5.5**) providing a quick and wide comparison between samples.

Results of artichoke metabolite fingerprinting were in agreement with those reported in literature [239]. It is worth to notice that guaianolides concentrations are much lower than phenolics ones, therefore in the same FancyTile two different colorimetric scales have been used for the different classes of compounds.

Recent data showed that mycorrhizal inoculation may represent an efficient and sustainable strategy to enhance plant biosynthesis of secondary metabolites [240]. Using the FancyTile the differences in metabolite profile between artichokes obtained from mycorrhized and not mycorrhized plants, can be easily appreciated and they are actually not very significant. As reported in **Figure 5.5**, not mycorrhized sample showed slightly higher concentration of feruloylquinic acid hexoside, but in mycorrhized artichoke higher concentration of gallic acid gallate and appearance of two phenolic acids not present in control sample (ferulic acid hexoside and gallic acid galloyl glucoside, namely) were observed. A similar trend was observed among flavonoids: not mycorrhized sample showed slightly higher levels of luteolin glucuronide glucoside and naringenin hexoside but in mycorrhized artichoke were observed higher concentration of quercetin malonyl galactoside and traces of two flavonoids not present in control sample (naringenin rhamnoside and apigenin glucosyl lactate, namely [224].

Quantitative and qualitative differences between samples were clearly detectable in guaianolides profile. In not mycorrhized artichokes grosheimin and its derivatives (deoxy and dihydroxy grosheimin) were the most abundant guaianolides and only little trace of cynaratriol was found. Mycorrhized artichoke showed higher level of total guaianolides than control sample: cynaratriol was the most abundant compound and traces of cynaropicrinin (not found in not mycorrhized control) were detected.

It has been suggested that modification of the secondary metabolite profile in response to biotic stress such as mycorrhizal colonization may be the consequence of a general plant defense response, which is later suppressed [241]. Generally, inoculation of plants results in an overall increase in the production of some new phenolic compounds during the progression of the infection [242]. However guaianolides and phenols are synthesized in response to different defense mechanisms and this could explain the different variations due to the mycorrhizal colonization. In particular, bitter guaianolides provide a significant barrier to herbivores, while phenolic compounds protect the plant from insect feeding [243].

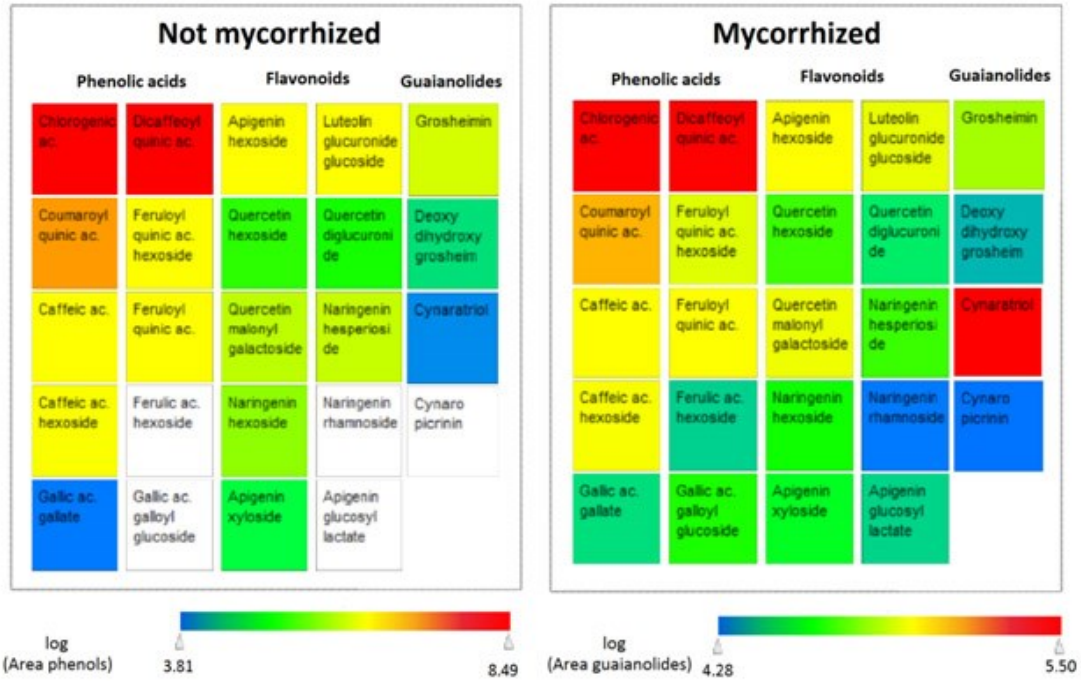

Figure 5.5: Example of FancyTile: phenolics and guaianolides comparison between mycorrhized and not mycorrhized artichoke

Data showed that, in different food matrices, FancyTile can be proposed as a comprehensive analytical procedure allowed a rapid and simultaneous detection of many compounds belonging to different chemical classes. By creating for each food an educated schema, an immediate comparison was possible highlighting only the differences among the relevant compounds for the specific material. This represent the main difference with the untargeted metabolomics approach providing a snapshot of all metabolites. However the enormous amount of data is often not the best way to obtain useful information; while FancyTiles could represent a tool for research programs aiming at specific improvements of foods, industrial processes, raw materials and ingredients.

Chapter 6

Discussion and future perspectives

6.1 Maillard reaction and chemometrics

The relationship between Maillard reaction and food quality does not exclusively rely on analytical aspects, even if the accurate quantification of intermediates, precursors and end products can provide an immediate snapshot of the impact of thermal treatments, hence of the final quality of foods. The overwhelming evidences on the relationship between Maillard reaction and food quality can be further investigated by following a chemometric approach and response surface methodology (RSM). Firstly, the impact of the carbonyls attachments and the consequent Strecker degradation for 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). These techniques can combine the monitoring of several classes of compounds (both precursors and end products) and their relationship with a plethora of technological interventions. Several examples are possible and the following paragraph represents a preliminary overview of the connection between analytical strategies, chemometrics and Maillard reaction.

6.2 Preliminary evidences on the link between MR and chemometric techniques

Preliminary PCA exploratory analysis have revealed that the presence of enzymes able to interfere in the Maillard pathways (i.e. asparaginase that oxidizes asparagine into aspartic acid and Faox that converts Amadori compounds into deoxyglucosone and free amino groups) is characterized by higher concentration of acetic acid, dicarbonyls and other autoxidation products likely deriving from the degradation of 1-deoxyglucosone. In this frame, it is possible to build a solid chemometric study able to classify the presence of the enzymes and its relationship to flavor development or to the MRPs formation. Secondly, the activity of enzymes should be investigated in relationship to the presence of free amino acids and Amadori compounds. In this respect, RSM can clarify the link between dose/response effects promoting the optimization of enzymes 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, Direct Analysis Real Time (DART-HRMS), 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 [244]. In particular, with proton transfer reaction mass spectrometry (PTR-MS) the study of volatiles formation during

the storage or to the thermal treatment it is possible to monitor the influence of the precursors on low molecular weight molecules formation. This technique has been widely used for the characterization of foods and for the monitoring of the volatiles [245]. PTR-MS enables the analysis of volatiles organic compounds (VOCs) at very low concentrations by direct injection. A soft chemical ionization is applied in this system by using $H3O^+$ ions as proton donors which can react with a wide variety of volatile compounds . The coupling of PTR-MS to TOF mass analyzers increases the sensitivity of the VOC analysis by detecting the concentrations at parts per billion per volume level (ppbv) with high mass resolution. $H3O^+$ is the mostly used ionization agent in PTR-MS studies [246]. Preliminary results of PTR-MS on low lactose milk, hierarchical clustering analysis (HCA) combined with PCA exploratory analysis revealed a direct influence of the proteolytic activity of lactase during storage and Maillard volatiles formation [247].

Despite the organization and visualization of data is important, the use of chemometric is the consequence when the activities of hundreds of molecules are measured, as a function of a specific treatment. Several methodologies have been introduced, among them the use of HCA and classification can be applied to distinguish the markers of the MR influenced by the use of some ingredients (i.e. preservatives and/or polyphenols), optimization of the thermal treatments, changes in the conditions of the storage and use of biochemical tools . 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 with the use of GC-MS or PTR-MS; B) the presence of Faox I and II and their de-glycating activity can be evaluated by monitoring the free Amadori compounds present in a specific food matrix [248]; C) the monitoring of free precursors and intermediates for the building of an heated map of tomato products.

In particular, in **Figure 6.1** the effects of thermal treatment on tomato products is evaluated by the quantitation of free amino acids and free APs. As revealed by the heated map, it is possible to distinguish among different products by taking into account free amino acids and APs. On the left part of the panel the double concentrated tomatoes are shown while moving from left to right it is possible to highlight minimally treated tomatoes (PC, PR and PCR). The color scale is directly imported from the FancyTiles approach, but in this case only the red scale is used, yellow cells

identifies a low ratio between free APs and free amino acids while the red cells identify high values of the ratio between the two groups.

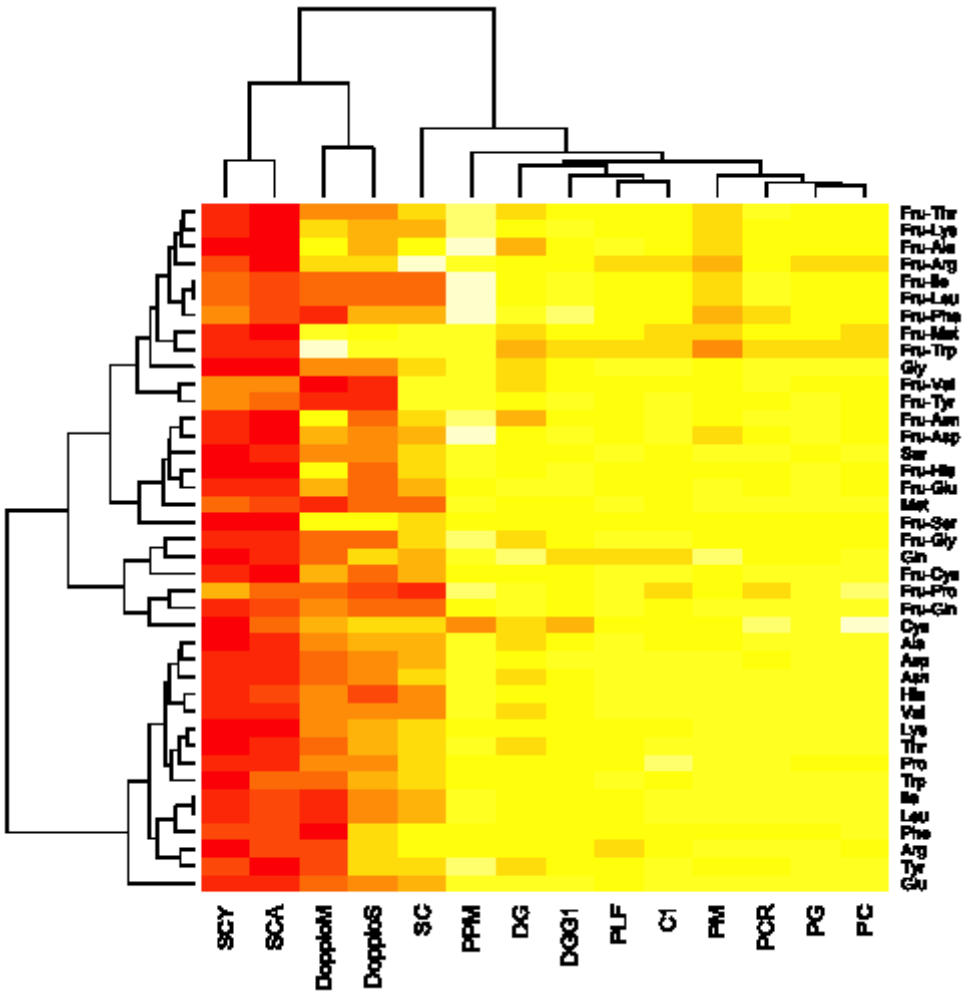

Figure 6.1: Heated map of a tomatoes sampling from the market. On the right y axis the variable identified, while the color identifies the ratio between free APs and free amino acids. On the top and on the left y axis the HCA is reported and the supervised recognition pattern can promote the identification of different groups.

6.3 Summary and main conclusions

The Maillard reaction supervises the quality of foods and its impact on the final acceptability of the consumers needs to be accurately undertaken by monitoring the key markers. In this respect, the fine tuning of the analytical strategies is a crucial aspect that concurs to the promotion of food quality. Four strategies have been evaluated in this thesis focusing on a the most important stages of the MR. In particular, moving from the analysis of precursors (i.e. amino acids) to the end products

(i.e. acrylamide) it is possible to pave the way for the implementation of chemometric techniques as a reliable tool for the modelling of quality through the MR.

In *chapter 1* a brief introduction of the relationship between Food quality and MR analytical techniques has been presented and an overview of the most used strategies has been presented.

In *chapter 2*, the first stages of the MR leading to the formation of the APs has been evaluated as an intriguing technique for the evaluation of the thermal impact on tomatoes and milk products by using ion pairing liquid chromatography coupled to HRMS. For the first time, the twenty fructose-amino acids were simultaneously quantified with free amino acids and the results obtained underpinned the use of the ratio between free APs and free amino acids as novel strategy for the evaluation of the Maillard impact on food quality.

In *chapter 3*, bound MRPs along with total lysine were quantified in several products and the method was systematically applied in industrial soybeans production. The use of stable isotope dilution assay (SIDA)/ solid phase extraction coupled to ion pairing liquid chromatography tandem mass spectrometry guaranteed the accurate and simultaneous quantification of the four markers. This technique combined the analysis of furosine (an intermediate) to the quantitation of CML, CEL and total lysine providing an immediate snapshot of nonenzymic browning of ε-side chain of lysine and on the loss of nutritive values of foods. The method was successfully applied in the monitoring of the MRPs formation of soybean products during the sterilization process, milk, breads and biscuits.

The accurate quantification of potentially toxic acrylamide by high resolution mass spectrometry was presented in *chapter 4*. The analysis of acrylamide in foods is one of the most challenging procedures in Chemistry and several drawbacks concur to define this complex scenario: the high polarity of acrylamide, its poor retention in common stationary phases, its reactivity, and the interferences of the matrix. The use of high resolution mass spectrometry defines new landscape in the accurate quantification of this small amide thanks to two main advantages the ability in delivering low-ppm mass accuracy up to 0.5 ppm and the possibility to reach high resolution. The use of HRMS can definitely promote the transferring of the acrylamide detection to routine analysis.

In *chapter 5* the FancyTiles approach was presented and the basis for its application in the MR were setup. In particular, beside the characterization and the comparison of products and nutritional quality, it was verified that this schema can be used also for the evaluation of the thermal damage.

Finally in *chapter 6*, future perspectives on the analysis of MRPs as a tool for the construction of solid chemometric models was presented with particular emphasis on two case study: the use of enzymes able to interfere in MR pathways and the monitoring of the MRPs formed; the use of polyphenols in the control of MR and the analysis of volatiles formed.

Abbreviations

- Maillard reaction (MR);
- Maillard reaction products (MRPs);
- Amadori products (APs);
- Mass spectrometry (MS);
- High Resolution Mass Spectrometry (HRMS);
- Solid phase extraction (SPE);
- Stable isotope dilution assay (SIDA);
- Ion pairing liquid chromatography (IPLC);
- Perfluoropentanoic acid (NFPA);
- Liquid chromatography (LC);
- Ultra-high pressure liquid chromatography (U-HPLC);
- Tandem mass spectrometry (MS/MS);
- Nuclear Magnetic Resonance (NMR),
- Supercritical Fluid Chromatography (SFC);
- Gas Chromatography (GC);
- Liquid Chromatography (LC).
- Olive oil mill wastewaters (OMWW);
- Full width half maximum (FWHM);
- Hydrophilic interaction liquid chromatography (HILIC);
- Nε-(2-Furoylmethyl)-L-lysine (furosine),
- Nε-(Carboxymethyl)-L-lysine (CML),
- Nε-(Carboxyethyl)-L-lysine (CEL),
- Direct Analysis Real Time (DART);
- Limit of detection (LOD);
- Limit of quantitation (LOQ);
- Multiple reaction monitoring (MRM);
- Single reaction monitoring (SRM);
- Time of flight mass spectrometry (TOF-MS);
- Fourier transform infrared spectroscopy (FT-IR);
- Proton Transfer Reaction (PTR).

Strategie analitiche per il controllo della qualità degli alimenti attraverso la reazione di Maillard

La reazione di Maillard occupa un ruolo fondamentale nella chimica degli alimenti. Lo studio della formazione di molecole quali acrilamide, CML, CEL, furosina e prodotti di Amadori può avere un impatto determinante nel controllo della qualità di alimenti trattati termicamente. In questa tesi sono state valutate quattro diverse strategie analitiche capaci di fornire un quadro generale non solo della concentrazione di alcuni marker specifici della reazione di Maillard, ma soprattutto di offrire un valido strumento per collegare intermedi di reazione e prodotti finali tipici alla qualità dell'alimento. Amino acidi e prodotti di Amadori sono stati monitorati attraverso l'utilizzo di spettrometria di massa ad alta risoluzione e, per la prima volta, il rapporto tra prodotti di Amadori ed amino acidi liberi è stato utilizzato come strumento per la valutazione del danno termico. I marker legati della reazione di Maillard (lisina totale, CML, CEL e furosina) sono stati quantificati mediante idrolisi acida, cromatografia di coppia ionica e spettrometria di massa tandem per valutare la glicazione in un' ampia gamma di alimenti. Infine l'analisi dell' acrilamide mediante spettrometria di massa ad alta risoluzione ha evidenziato come sia possibile coniugare la calibrazione in continuo di uno spettrometro di massa alla presenza di alcuni contaminante alfine di rendere l'analisi di piccole molecole precisa ed accurata anche in cromatografia liquida. Infine lo sviluppo delle FancyTiles ha aperto un nuovo scenario: la combinazione della quantificazione dei prodotti della Maillard con tecniche di chemometrica utili a fornire un quadro dettagliato della qualità degli alimenti. I risultati ottenuti hanno dimostrato l'importanza del monitoraggio dei prodotti della reazione di Maillard al fine di garantire la qualità degli alimenti.

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