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INNOVATIVE STRATEGIES FOR THE MITIGATION OF ACRYLAMIDE CONTENT IN DIFFERENT FOOD PRODUCTS

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Abstract

Acrylamide (AA) is an undesired food toxic compound classified as “probably carcinogenic to humans” (group 2A) by the International Agency for Research on Cancer due to its toxicological effects including neurotoxicity, genotoxicity, carcinogenicity, and reproductive toxicities. In the last few years, authorities and regulations have been more restrictive concerning the maximum AA levels allowed in heat-treated foods and beverages. The latest Commission Regulation (EU) 2017/2158 contains benchmark levels and measures to reduce AA levels in different food groups and subgroups that contribute to the higher dietary intake. AA is principally formed during food thermal processing (at a temperature above 120 °C) by the reaction of free amino acids with the carbonyl group of reducing sugars as part of the Maillard reaction. Food heat treatments (frying, baking, roasting, etc.) are extremely important for the transformation and sanitation of many raw materials, semi-finished and formulated products which, through these processes, reach the desired levels of shelf-life and sensorial properties. Nevertheless, the same treatments are often responsible for the reduction of their nutritional properties and the formation of some toxic compounds such as AA.

The aim of this PhD research project was to identify, characterise and optimise some AA mitigation strategies in the most at-risk widely consumed foods such as potato, coffee, and bakery products. Some AA control strategies were selected and investigated for each food category, also considering the main quality characteristics of the final products, in order to adopt a holistic risk/benefit approach.

The comprehensive results obtained during the three years of research activity have allowed a deeper knowledge of the traditional and innovative AA mitigation strategies investigated, which can be extremely promising and useful for both the food industry and international authorities. The most promising strategies studied in terms of reduction of AA while maintaining the main quality characteristics of the examined food products were: the application of pulsed electric fields and yeast immersion as pre-treatments of chips for frying; the selection of high roasting degrees for coffee products; the selection of static baking conditions for biscuits; the optimisation of alternative biscuit products’ formulations by both the use of chickpea legume flour and of flour from bean with intact cotyledon cell walls.

List of publications

The present thesis is based on the following original published and submitted papers, which are referred to in the main text by their Roman numerals:

I. **Schouten M.A.**; Tappi S.; Romani S. (2020). Acrylamide in coffee: formation and possible mitigation strategies - A review. *Critical Reviews in Food Science and Nutrition*, 60, pp. 3807-3821 (Available online 5 January 2020, doi.org/10.1080/10408398.2019.1708264).

II. **Schouten M.A.**; Genovese J.; Tappi S.; Di Francesco A.; Baraldi E.; Cortese M.; Caprioli G.; Angeloni S.; Vittori S.; Rocculi P.; Romani S. (2020). Effect of innovative pre-treatments on the mitigation of acrylamide formation in potato chips. *Innovative Food Science and Emerging Technologies*, 64, August 2020, 102397, pp. 1-11 (Available online 29 May 2020, doi.org/10.1016/j.ifset.2020.102397).

III. **Schouten M.A.**; Tappi S.; Angeloni S.; Cortese M.; Caprioli G.; Vittori S.; Romani S. (2021). Acrylamide formation and antioxidant activity in coffee during roasting - A systematic study. *Food Chemistry*, 343, 1 May 2021, 128514, pp. 1-9 (Available online 1 November 2020, doi.org/10.1016/j.foodchem.2020.128514).

IV. **Schouten M.A.**; Tappi S.; Glicerina V.; Rocculi P.; Angeloni S.; Cortese M.; Caprioli G.; Vittori S.; Romani S. (2021). Formation of acrylamide in biscuits during baking under different heat transfer conditions. *LWT – Food Science and Technology*, 153, 112541, pp. 1-8 (Available online 27 September 2021, doi.org/10.1016/j.lwt.2021.112541).

V. **Schouten M.A.**; Tappi S.; Rocculi P.; Romani S. (2021). Mitigation Strategies to Reduce Acrylamide in Cookies: Effect of Formulation. *Food Reviews International*, pp. 1-41 (Available online 6 February2022, doi.org/10.1080/87559129.2021.2023171).

VI. **Schouten M.A.**; Fryganas C.; Tappi S.; Romani S.; Fogliano V. (2022). The use of chickpea flour is an effective formulation strategy to reduce the acrylamide formation in biscuits. Manuscript submitted for publication (11 February 2022).

VII. **Schouten M.A.**; Fryganas C.; Tappi S.; Romani S.; Fogliano V. (2022). The use of kidney bean flour with intact cell walls reduces the formation of acrylamide in biscuits. Manuscript submitted for publication (18 January 2022).

Thesis aim and outline

This PhD research project aimed to identify, characterise and optimise some acrylamide (AA) mitigation strategies in the most at-risk widely consumed food products such as potato, coffee and bakery products. The best AA control strategies were investigated for each food category, also in order to obtain the optimal quality characteristics of each final product, adopting a holistic risk/benefit approach. Comprehensive and systematic studies were carried out to assess the effect of each applied process on the AA formation and on the main quality characteristics of each food product category. A schematic overview representation of the thesis outline is presented in **Figure 1**.

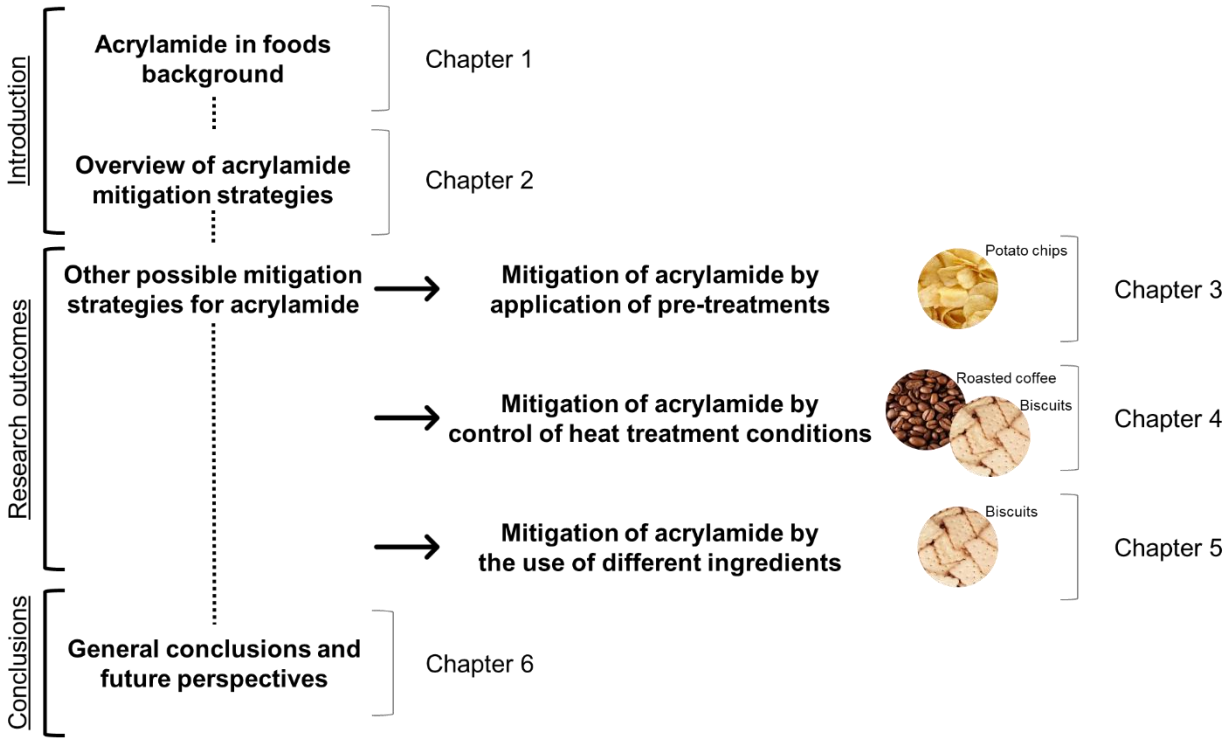


Figure 1 - Structure of the thesis chapters.

In particular:

Chapter 1 provides a general introduction to the background of AA in foods by giving a thorough review of the mechanisms involved in its formation; its physicochemical characteristics; its effects on human health and the status of the current legislation and regulations.

Chapter 2 explores the different AA mitigation strategies studied so far along the production stages of potato chips, coffee and bakery products. For roasted coffee

products, the AA mitigation strategies are limited, the most promising being those related to raw material selection and modification of conventional roasting process conditions. For fried potato and bakery products, strategies involving changes in formulation and the application of unconventional processing techniques are also promising. The drawbacks of each type of strategy were also discussed.

Chapter 3 investigates the effects of innovative pre-treatments to reduce AA in fried potato products, such as pulsed electric fields and yeast *A. pullulans* immersion, alone and in combination for different application times. The AA and its precursors (e.g. reducing sugar and asparagine) contents, quality parameters, such as moisture, oil content, colour and texture, of potato crips were also assessed.

Chapter 4 aims to develop a study on the management of heat-treatment conditions to control AA levels in roasted coffee and baked biscuits. The effect of the roasting process of Arabica and Robusta green coffee on the development of both toxic and some beneficial antioxidant compounds was investigated. This study aimed at assessing how the heat treatment can be directly linked to the presence of unhealthy compounds, such as AA, and healthy compounds, among which trigonelline, nicotinic and caffeoylquinic acids. Moreover, the influence of two commonly used baking methods, such as conduction and forced air convection, on the AA formation in biscuits was evaluated. The AA precursors (asparagine, glucose and fructose) and some quality characteristics (weight loss, moisture, water activity, texture and colour) were analysed in biscuits during baking to identify the optimal baking conditions to obtain the lowest AA level and at the same time acceptable quality characteristics in the final product.

Chapter 5 studies the effects of formulation to reduce AA in biscuit products. In a first trial, the potential of the food matrix in biscuits prepared with different flours by standardizing the initial asparagine content in the formulations was investigated. The biscuits were formulated by partially replacing wheat flour with protein-rich legume flours from lupins and chickpeas. Starting with the same level of asparagine in all biscuit formulas, the structure-related effects of the product on AA formation during baking were investigated. The lupin, chickpea and wheat flours were characterized for their composition and their physical properties. Biscuit samples after baking were investigated for the AA levels and for the AA precursors contents, as well as for their main quality

characteristics. Subsequently, the effect of legume ingredients with intact and broken cotyledon cell walls on AA formation in biscuits during baking was examined. Biscuit doughs were prepared by partially replacing the wheat flour with red kidney beans as a mash, obtained by boiling and mashing the beans, or as flour, prepared by grinding the beans with a cryo-mill. The integrity or non-integrity of the bean cell walls in the obtained flour and mash were analysed by scanning electron microscopy. Bean ingredients, raw biscuit doughs and baked biscuits were evaluated for the content of AA and its precursors, in addition, baked biscuits were also characterised for the main quality attributes.

Chapter 6 summarises the main findings of all the research activities carried out and reported in the previous chapters; moreover, further research directions were proposed.

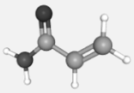
Chapter 1

Introduction: acrylamide in foods background

1.1 Chemical proprieties, toxicity and formation pathways

Acrylamide (AA), also called 2-propenamide, is an unsaturated amide and associated to the Chemical Abstracts Service registry number 79-06-1. **Table 1.1** shows the chemical structure and the main physical properties of this compound. It is a low molecular weight, white, odourless, low volatile and crystalline solid at room temperature and its boiling and melting temperatures are 125.0 °C and 84.5 °C, respectively. Its density is 1.27 g/mL at 25 °C. AA is highly soluble in water (2155 g/L at 30 °C) and other polar solvents such as methanol (1550 g/L), ethanol (862 g/L) and acetone (631 g/L), on the other hand, it is less soluble in non-polar solvents including chloroform (26.6 g/L) and benzene (3.46 g/L) (Šimko & Kolarič, 2019). As it can be noticed from its chemical structure, AA is a difunctional monomer with conjugated double bonds; the functional group that determines its chemical characteristics is the amide, i.e. the -NH_2 bonded to the carbonyl carbon. This molecule is characterised by electrophilic reactivity and can therefore react with nucleophilic groups of various biological molecules (Šimko & Kolarič, 2019). AA lacks a strong chromophore for UV detection and does not fluoresce, in addition, exhibits both weakly acidic and basic proprieties (Matoso et al., 2019).

Table 1.1 - The main physical and chemical characteristics of acrylamide.

Characteristics	Value
Molecular structure	
Molecular formula	C_3H_5NO
IUPAC name	prop-2-enamide
Molecular weight (g/mol)	71.08
Melting point (°C)	84.5
Boiling point (°C)	125.0
Density at 25 °C (g/mL)	1.27
Water solubility at 30 °C (g/L)	2155
Vapor pressure at 25 °C (mm·Hg)	0.007
Specific gravity at 30 °C (kg/dm^3)	1.122

Since 1950, AA is usually commercially produced from the hydration of acrylonitrile by sulfuric acid monohydrate at 90 or 100 °C. The presence of polar amide group and a vinyl

function in AA monomers allows a rapid polymerization leading to the formation of polyacrylamide. This polymer is commonly used in chromatography and electrophoresis in research laboratories and several industrial applications such as wastewater treatment as a flocculant, in the pulp and paper industry to improve paper quality and in the cosmetics and textile industry (Albedwawi et al., 2021; Maan et al., 2020; Matoso et al., 2019; Tepe & Çebi, 2019).

In 1994, the International Agency for Research on Cancer (IARC), established the toxicity of the AA monomer, classifying it in group 2A as a 'probable human carcinogen' (IARC 1994). The scientific community became more and more interested in AA also after an environmental disaster during the construction of tunnels for high-speed railways in Sweden (1997), in which workers were exposed to AA-containing sealants during the accident (Matoso et al., 2019). The presence of AA-hemoglobin adducts, considered as a bioindicator of AA presence, was assessed in some tunnel worker blood samples and compared with a control group. A clear dose-response was found between the Hb-adduct levels and deteriorated symptoms of the peripheral nervous system (PNS) that were reversed 18 months after cessation of exposure (Hagmar et al., 2001; Matoso et al., 2019).

The toxicological characteristics of AA including genotoxicity, neurotoxicity, carcinogenicity, hepatotoxicity and reproductive toxicity have been extensively investigated by animal studies (Crawford et al., 2019; Mollakhalili-Meybodi et al., 2021; Perera et al., 2021; Pundir et al., 2019). Several kinds of research have shown that AA is absorbed and rapidly distributed systemically into the bodies of humans and animals through ingestion, inhalation and skin contact. After absorption, AA is rapidly distributed to many tissues and organs through the bloodstream, leading to the detection of this compound in the brain, heart, liver, thymus, kidneys and breast milk via body stream circulation (Eisenbrand, 2020; Matoso et al., 2019; Mollakhalili-Meybodi et al., 2021). In mammalian cells, AA is mainly metabolised by conjugation with glutathione either non-enzymatically or by glutathione-S-transferase enzyme and by epoxidation reaction mediated by cytochrome P-450 CYP2E1 enzyme (Eisenbrand, 2020; Hartwig et al., 2020; Koszucka & Nowak, 2019; Kwolek-Mirek et al., 2011). The metabolite of the epoxidation reaction is glycidamide, a highly genotoxic reactive metabolite which, together with AA itself, can react with macromolecules such as haemoglobin (i.e. specific adducts AA-Hb or GA-Hb), DNA, serum albumin and enzymes to form toxic and carcinogenic adducts. Both AA and glycidamide are excreted in urine as mercapturic acids (Crawford et al.,

2019; Eisenbrand, 2020; El-Zakhem Naous et al., 2018; Pundir et al., 2019; Sarion et al., 2021). A considerable amount of research has been conducted on the toxic effects of AA exposure in different experimental models leading to highlight the risk in the peripheral and central nervous system (neurotoxicity) as well as mutagenicity, genotoxicity, carcinogenicity, toxicity effects regarding reproductive and developmental abilities in both human and animals (Aras et al., 2017; Bowyer et al., 2008; Duan et al., 2015; Erdemli et al., 2016; Ghanayem et al., 2010; Hamdy et al., 2012; Hułas-Stasiak et al., 2013; Kermani-Alghoraishi et al., 2010; Jia Li et al., 2016; Ma et al., 2011; Pan et al., 2015; Pourentezari et al., 2014; Şen et al., 2015; H. Wang et al., 2007, 2010; Wei et al., 2014; Zargar et al., 2016; Zhao et al., 2017). However, further studies are needed to clarify in detail the effects, molecular mechanisms of action and consequences for human health (Matoso et al., 2019).

All these findings revealed a considerable increase in the investigation of AA as a potentially dangerous substance for humans. In 2002, the Swedish National Food Administration (SNFA) and Stockholm University discovered the presence of high levels of this toxic compound in a wide range of common foods (Tareke et al., 2002). Since this discovery and related negative effects on human health, much attention has been focused on investigating the various factors involved in the presence of AA in some foods. As a result, it has been established that AA is not a component of foods but is one of several so-called process-related contaminants occurring in heat-processed foods.

Numerous mechanisms of AA formation have been extensively elucidated, finding that AA levels can depend mainly on factors such as processing temperature and time, cooking method, variety of raw materials, pH, product thickness and water activity (Dal Castel Krein et al., 2020; Pundir et al., 2019). However, although AA formation has been extensively reviewed in the literature, the process is not completely understood (Albedwawi et al., 2021). A summary of the various major and minor pathways proposed for AA formation is observed in **Figure 1.1**.

It is well-established that the main mechanism leading to the formation of AA in food is the Maillard reaction, a complex series of reactions also known as the non-enzymatic browning reaction, which occurs in food products during common cooking methods such as baking, frying, grilling and roasting at high temperatures (> 120 °C). The series of reactions may follow multiple and often simultaneous pathways, the same type of molecule may pass through a variety of fates, some alternative to each other, some parallel, until the final generation of an extremely complex pool of reaction products.

Overall, besides AA formation, the Maillard reaction plays an important role in foods because it influences multiple quality parameters, including organoleptic properties, colour and nutritional value by contributing to both desired and undesired sensorial changes which affect consumers' acceptance (Altunay et al., 2018; Baskar & Aiswarya, 2018; Lund & Ray, 2017). The formation of AA through the Maillard reaction involves the condensation reaction between the carbonyl group of reducing sugar (e.g. glucose and fructose) with an amino residue of amino acid, mainly asparagine, to produce the Schiff base, which might be transformed into Amadori compounds or decarboxylated to azomethine ylide (Khorshidian et al., 2020; Rifai & Saleh, 2020; Stadler et al., 2002). AA can be formed through β-elimination of decarboxylated Amadori compound or directly from azomethine ylide or its hydrolysis followed by the deamination of 3-aminopropionamide (3-APA) (Khorshidian et al., 2020; Maan et al., 2020; Pedreschi et al., 2014; Rifai & Saleh, 2020; Stadler et al., 2002). It has been reported that fructose increases the AA content by about two times in comparison with other reducing sugars because it contains two α-hydroxylic groups rather than one as is the case with other sugars such as glucose (Perera et al., 2021). Furthermore, certain other carbonyl compounds, such as 2-deoxyglucose, 2,3-butanedione, octanal and decanal, can react with asparagine via the Maillard reaction instead of reducing sugars (Khorshidian et al., 2020; Knol et al., 2010; Krishnakumar & Visvanathan, 2014; Maan et al., 2020; Perera et al., 2021).

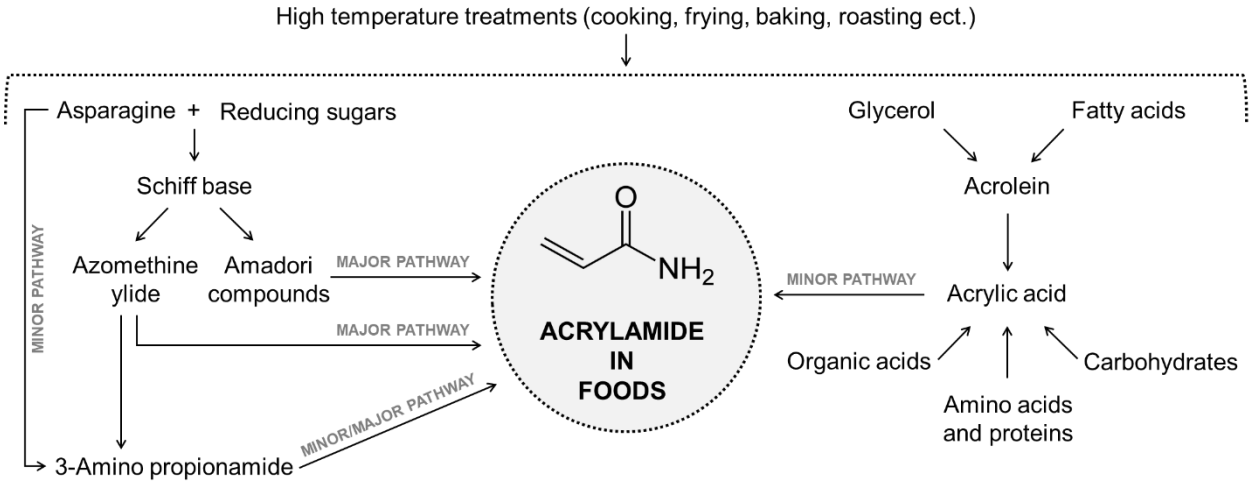


Figure 1.1 - Simplified overview of the possible pathways of acrylamide formation in foods.

Even in the absence of reducing sugars or carbonyl compounds, AA can be formed 3-APA can be generated from simple asparagine by direct decarboxylation and

deamination reactions at high temperatures (Perera et al., 2021; Zyzak et al., 2003). Nevertheless, the high process temperatures lead to its rapid intramolecular cyclization, making this pathway of AA formation of limited importance (Granvogl & Schieberle, 2006; Perera et al., 2021; Yaylayan et al., 2003).

In addition, in the absence of asparagine, another possible mechanism for AA formation involving acrolein as a precursor has also been stated (Krishnakumar & Visvanathan, 2014). Acrolein can be mainly formed during heat treatment from glycerol via heterolytic acid-catalysed carbonium ion mechanism or from the oxidative degradation of fatty acids above their smoke point, after which it can be converted to acrylic acid (Das & Srivastav, 2012; Liu et al., 2015; Maan et al., 2020; Perera et al., 2021). Acrylic acid may also be obtained from the thermal decomposition of amino acids and proteins, degradation of carbohydrates and decarboxylation of an organic acid such as lactic acid, citric acid and malic acid (Das & Srivastav, 2012; Perera et al., 2021). The formed acrylic acid can subsequently react with ammonia, released during thermolysis of amino acids, to form AA (Yaylayan & Stadler, 2005). The formation of AA from acrolein and acrylic acid is limited to the availability of free ammonia in food, which is why its formation in food from acrylic acid appears to be only of marginal importance (Guenther et al., 2007; Perera et al., 2021).

1.2 Occurrence, intake and health risk

The occurrence of AA, due to the presence of precursors, has been extensively reported in a vast range of foods such as potato-based products (e.g. potato chips and French fries), cereal-based products (e.g. bread, biscuits, breakfast cereals and baby foods), roasted coffee, roasted nuts and seeds (e.g. hazelnuts and almonds), meat-based products (e.g. breaded fried meat), tea as well as processed fruits and vegetables (Capuano & Fogliano, 2011; Eslamizad et al., 2019; Khorshidian et al., 2020; Maan et al., 2020; Nematollahi et al., 2021). Examples of AA range levels for different types of food are given in **Table 1.2**. Besides foods, AA is also a component of tobacco smoke, nevertheless, it is of marginal importance as it is only one of the main issues in the use of tobacco products (Raffan & Halford, 2019; Schettgen et al., 2003).

It has been established that the adverse health effects of AA manifest with prolonged intake and are directly related to the frequency of consumption, therefore is commonly consumed in most countries of the world and, having a high AA content, potato, cereal

and roasted coffee products are the food groups that most contribute to AA exposure (Maan et al., 2020; Nematollahi et al., 2021; Šimko & Kolarič, 2019). According to some literature research, the source of dietary AA in fried potatoes accounts for 20-5200 µg/kg.

Table 1.2 - Examples of acrylamide range levels in some common foods.

Food sample	Content (µg/kg)	Reference
Potato products		
Potato chips	108-2180	(Mesías & Morales, 2016b)
	173-3444	(Esposito et al., 2017)
French fries	59-5200	(Paul et al., 2016)
	20-4000	(Mesías, Nouali, et al., 2020)
Cereal products		
Bread	<LOQ-695	(Gündüz & Cengiz, 2015)
	14-59	(Crawford et al., 2019)
Biscuits	18-72	(Žilić et al., 2020)
	155-661	(Chen et al., 2020)
Breakfast cereals	62-803	(Rufián-Henares et al., 2006)
	<LOD-810	(Capei et al., 2015)
Coffee products		
Roasted coffee	130-470	(Lachenmeier et al., 2019)
	109-730	(Paper III)
Espresso drink	11-36 µg/L	(Soares et al., 2006)
	23-390 µg/L	(Alves et al., 2010)
Instant drink	42-338 µg/L	(Şenyuva & Gökmen, 2005)
	16-79 µg/L	(Başaran et al., 2020)
Other products		
Baby foods	2-516	(Tateo & Bononi, 2003)
Chocolate	102	(Pardo et al., 2007)
Tomato sauce	50-124	(Tateo et al., 2007)
Roasted peanuts	11-37	(Esposito et al., 2017)
Green tea	10-23	(Khan et al., 2017)
Black tea	62-97	(Khan et al., 2017)
Hazelnuts	19	(Tepe et al., 2020)

LOQ: Limit Of Quantification; LOD: Limit Of Detection.

The AA range for bakery products and coffee is 14-810 and 11-730 µg/kg, respectively (**Table 1.2**). Among these principal food groups, even though AA concentration in coffee beverages is relatively low (e.g. 11-390 µg/L), it is considered the major contributor to AA exposure in adults because of the high amounts of coffee consumed (Mojska & Gielecińska, 2013). On the other hand, it is suggested that the intake of foods with high levels of AA is significantly higher in the diets of children and adolescents who have a higher consumption of foods rich in carbohydrates, such as French fries and potato chips (Albedwawi et al., 2021; Normandin et al., 2013). However, it is important to take into account that the AA contents in the same food type can highly vary due to the difference in raw materials composition, formulation, process techniques and storage conditions. There are also differences in AA levels among home-cooked, factory-produced and restaurant food (Albedwawi et al., 2021). All these factors, together with different individuals cooking and eating habits, have resulted in variable estimates of dietary exposures of AA among populations of various areas and across time (Maan et al., 2020; Timmermann et al., 2021).

The estimated average range of intake of dietary AA by an average consumer can be considered to be about 0.4 µg/kg body weight per day (bw/d), although consumption can generally vary from 0.3 µg/kg bw/d to 5 µg/kg bw/d (Busk, 2010; Perera et al., 2021; Rifai & Saleh, 2020; Stadler & Lineback, 2009). The average value is defined aiming at the consumption of an adult (> 18 years) population; however, it is suggested that the intake of foods with high levels of AA (i.e. fried and ready-to-eat foods) is considerably higher in the diet of adolescents (11-17 years) and children (3-10 years) identifying these as the age groups most vulnerable to exposure to AA (Dybing et al., 2005; European Food Safety Authority, 2015; Normandin et al., 2013).

The AA does not have a reliably identifiable "*safe dose*", which means that low-dose exposure may be followed by a period of symptomatic silence in which the harmful effects of the chemical may not be clinically evident, but morphological and/or biochemical changes may be present (Adewale et al., 2015; European Food Safety Authority, 2015; Rifai & Saleh, 2020). Meanwhile, the indicative maximum tolerable daily dose for cancer development or other potential adverse effects for a man weighing 60 kg has been estimated at 2.6 and 16 µg/kg per day based on AA or glycidamide, respectively (Albedwawi et al., 2021; European Food Safety Authority, 2015; Rifai & Saleh, 2020; Tardiff et al., 2010). Furthermore, according to a study conducted by DSM's Global Insight Series, only 22% of consumers surveyed in France, the UK and the US are aware of AA-

related health problems, while in Germany this figure reaches 54%. In general, those who are aware expect their country's manufacturers and regulations to take action to reduce the AA content in food products (Green, 2018; Ostermeier et al., 2020).

1.3 Legal regulations, benchmark levels and guidelines

Since AA is present in many different types of food, it is evident that the likelihood of reaching an intake level dangerous to human health is high, so the need for combined action by governments, industry and scientists has increased since its discovery.

In 2005, the European Foods Safety Authority (EFSA) Scientific Panel on Contaminants in the Food chain (CONTAM) recognizes the presence of AA in food and together with food industry operators, specialists and researchers from member countries have researched AA training pathways and developed a set of measures to reduce the level of this contaminant in food. Thus, in 2007, the European Commission (EC) published the Recommendation 2007/331/EC on monitoring the level of AA in food which provides for a three-year monitoring program (2007-2009) of AA in certain foods, in order to offer concrete information on foodstuffs that have a high level of AA or those that by consumption high food have a significant contribution to its assimilation. The categories of foods covered by the monitoring program are French fries (sold as ready-to-eat), potato chips, pre-cooked potato products for home cooking, bread, breakfast cereals, biscuits (including infant biscuits), roasted coffee, jarred baby foods (potato, root vegetable or cereals) and other products (e.g. potato rösti, gingerbread, coffee substitutes) (European Commission, 2007). The Recommendation 2007/331/EC requires that sampling by member states to be carried out at different market levels (e.g., supermarkets, smaller shops, bakeries, restaurants, etc.). A minimum number of samples were required for each product category, for each country and a unitary model for reporting the types of food taken and the results obtained.

In 2009, EFSA published data from its 2007-2009 monitoring of AA in a scientific paper, concluding that there was "*no consistent trend among food groups towards lower levels of acrylamide*" (European Food Safety Authority, 2009).

Subsequently, in 2010, as it was found that the monitoring programme for food classification needed to be improved, the EC issued the new Recommendation 2010/307/EC on monitoring the level of AA in food which included a revised categorisation of food products (European Commission, 2010). In the same year a second EFSA report

entitled "*Update on acrylamide levels in food from monitoring years 2007-2010*" concluded that a trend towards lower levels of AA was becoming apparent but that it was limited to certain food groups, with levels in most food groups remaining unchanged and, in a few food categories, showing a rise (European Food Safety Authority, 2010). In agreement with the results, the ESFA considers it is essential to continue collecting data for annual monitoring of AA levels in foods, as well as to continue research into how AA is formed and how to eliminate it by food businesses operators and Member States authorities. This led to EC Recommendation on investigations into the levels of AA in food of 10.1.2011 (document C (2010) 9681 final), which introduced the concept of indicative values for AA in foods (European Commission, 2011). The indicative values that were set for some food categories are given in **Table 1.3**. These EC indicative values were not intended as regulatory limits or safety thresholds; but they were set for different food categories at levels that the food industry should be able to achieve based on EFSA's 2007-2008 monitoring data (European Commission, 2011).

Further EFSA reports, followed in 2011 and 2012, found small changes in AA levels over the monitoring period, with some product categories showing a small decrease but others, including crispbread, French fries from fresh potatoes and instant coffee showing an increase with 3% and 30% of samples in different food categories exceeding the EC indicative value set for that product type. According to the new monitoring data, the EC's response was a Recommendation 2013/647/EU (European Commission, 2013), which revised and reduced indicative values for many food types as well as improved the food products categorization introducing some subcategories (**Table 1.3**).

This was followed in 2015 by the publication of EFSA's CONTAM Panel's assessment of the public health risk of dietary AA, confirming that consumption of AA potentially increases the risk of developing cancer for consumers in all age groups as it is present in a wide range of food products (European Food Safety Authority, 2015).

Continued efforts are needed to reduce exposure to AA, consequently, the EC has undertaken another process of strengthening its risk management regulations for AA by issuing Commission Regulation (EU) 2017/2158 which is currently in force since 11 April 2018 (European Commission, 2017). This regulation used stronger language, replaced indicative values with "*benchmark levels*", again, these levels are not yet regulatory limits but are described as performance indicators rather than triggers for investigation and substantially lower than the corresponding indicative value for almost all product types (**Table 1.3**). For instance, compared to the 2013 indicative values, the maximum value

was reduced substantially by 17, 25 and 30% for French fries, potato chips and biscuits respectively, while a smaller reduction of 13% was applied to roasted coffee.

Table 1.3 - Indicative values and benchmark levels for acrylamide in foods according to European Commission (EC) Recommendations of 10.1.2011, 2013/647/EU and Regulations 2017/2158/EU (Adopted from Raffan and Halford, 2019).

Food	Indicative value set in 2011 (µg/kg)	Indicative value set in 2013 (µg/kg)	Benchmark level set in 2017 (µg/kg)
French fries	600	600	500
Potato chips	1000	1000	750
Soft bread (wheat)	150	80	50
Soft bread (other)	n.r.	150	100
Breakfast cereals: bran products, whole grain cereals, gun puffed grain	400	400	300
Breakfast cereals: wheat and rye-based	n.r.	300	300
Breakfast cereals: maize, oat, spelt, barley and rice-based	n.r.	200	150
Biscuits and wafers	500	500	350
Crackers	500	500	400
Crispbread	500	450	350
Gingerbread	n.r.	1000	800
Cereal-based baby foods	100	50	40
Baby foods (not cereal-based) without prunes	80	50	n.r.
Baby foods (not cereal based) with prunes	n.r.	80	n.r.
Biscuits and rusks for infants and young children	250	200	150
Roast coffee	450	450	400
Instant coffee	900	900	850
Coffee substitute (cereal-based)	n.r.	2000	500
Coffee substitute (chicory)	n.r.	4000	4000

n.r.: not reported in the EC Recommendation or Regulation.

In addition to benchmark levels, the Regulation 2017/2158/EC lays down mitigation measures to reduce AA formation to be taken by food business operators for each food category, from variety selection through crop management to a range of measures that are effective during food processing. These are effectively codes of practice, and the wording of the Regulation makes it clear that the adoption of these measures is compulsory, in fact, food business operators "shall" apply the measures that are set out to reach the lowest possible levels of AA below the reference levels established in this normative act. Details of the mitigation measures taken must be provided to the authorities if the products exceed the benchmark levels. Another key aspect of the Regulation is the obligation for all food business operators to monitor AA levels in their products, even if the businesses carry out retail activities and/or supply directly; only local businesses are exempted from this obligation. Moreover, Member States are required to ensure compliance with this regulation (European Commission, 2017). As it was concluded that Regulation 2017/2158/EC did not present adequate available data on the presence of AA in foods, Recommendation 2019/1888/EC on monitoring the presence of AA in certain foods was adopted in 2019 by the European Commission (European Commission, 2019). This normative act includes a new list of non-exhaustive food products that must be monitored in order to detect risks and implement additional prevention and/or reduction measures against this contaminant. The new food list adds several specialties products in the potato, bakery, cereal products categories and other products including vegetable crisps/fries, roasted nuts, roasted oilseeds, dried fruits, roasted cocoa beans and derived cocoa products, olives in brine, coffee substitutes not based on chicory or cereals, fudge, caramel and nougat.

In conjunction with EFSA and EC monitoring and regulation of AA, since 2005 the FoodDrinkEurope organization, who represents economic operators in the European food and beverage industry, developed a set of "*acrylamide toolbox*" tools for industrial food application. The toolbox includes the most recent and relevant information from authorities, scientists, international research organizations and economic operators, about the ways of AA formation and the approaches of reducing it from some food products providing practical guidelines. This tool is updated periodically, the 15th and latest version was published in 2019 taking into account product categories such as "*potato-based snacks*", "*French fries and other cuts, deep-fried, potato products*", "*cereal/grain-based products (fine bakery wares and breakfast cereals)*", "*fine bakery wares (biscuits, crackers, wafers, crispbread and gingerbread)*", "*breakfast cereals*",

"*coffee*", "*coffee substitutes (mainly based on cereals and chicory)*", "*baby biscuits, infant cereals and baby foods other than cereal-based foods*". The material presents 14 different parameters ("*tools*"), organized into four broad categories ("*toolkit compartments*"): agronomic factors, manufacturing recipe (basic formula, ingredients, product form), food processing method and final preparation. These "tools" can be used alone and in combination by the food industry, depending on the specific needs of each to reduce the level of AA in their products (FoodDrinkEurope, 2019; Sarion et al., 2021).

The European Union is probably at the forefront of developing a regulatory framework for the presence of AA in foods. Until now, there has been less active in the United States, at least at the Federal level. The Food and Drug Administration (FDA), for example, has not introduced anything equivalent to the EC's indicative values or benchmark levels for the control of AA, although it has issued an "action plan" with the objectives of developing screening methods, assessing dietary exposure and identifying methods to reduce it. In 2016, the FDA developed a "Guidance for Industry" that outlines strategies to help growers, manufacturers and foodservice operators reduce AA in the food supply. And for consumers, the FDA has developed resources that contain information about AA and ways to reduce exposure from foods prepared at home (Food and Drougs Administration, 2016). A further example involves a state authority acting; in 2005, the Attorney General of the State of California filed a lawsuit against five potato chips and French fries manufacturers along with four fast-food chains for failing to label their products with a Proposition 65 warning to alert consumers to the presence of AA. Proposition 65 is California's Safe Drinking Water and Toxic Enforcement Act, which requires companies to post warnings about all compounds in their products that can cause cancer. The lawsuit was settled in 2008 when manufacturers pledged to reduce the level of AA in their products and fast-food chains agreed to display AA warnings in their restaurants. Then, in 2010, the Council for Education and Research on Toxics (CERT), a small non-profit organisation, filed a lawsuit under Proposition 65 against more than 90 food companies (especially coffee product manufacturers), demanding that they remove AA from their products or warn consumers of the presence of AA through warning signs and/or labels. In this case, the California Office of Environmental Health Hazard Assessment moved to exempt coffee from Proposition 65 warnings (Raffan & Halford, 2019).

Other countries where regulators have taken a stand include Canada, where AA has been added to the list of chemicals in the government's Chemicals Management. Health Canada has implemented an AA monitoring programme to assess the effectiveness of

the AA reduction strategies it recommends and to evaluate their implementation by the food industry, with the possibility of setting some "*reduction targets*" in the future. Food Standards Australia New Zealand (FSANZ), Japan and Hong Kong authorities have also taken similar positions (Raffan & Halford, 2019).

Chapter 2

Introduction: overview of acrylamide mitigation strategies

2.1 Mitigation approaches

The discovery of high levels of AA in many foods has for years called for its content to be reduced, thus reducing its intake. Research and studies have focused on identifying and developing possible strategies to reduce AA levels in thermally treated foods (i.e. potato, bakery and coffee products) through the process (Medeiros Vinci et al., 2012; Sarion et al., 2021; **Paper I**; **Paper V**). Various traditional and more innovative strategies to reduce AA levels in foods such as potato, coffee and bakery products have been widely studied in the literature, which can be explained as shown in **Figure 2.1**.

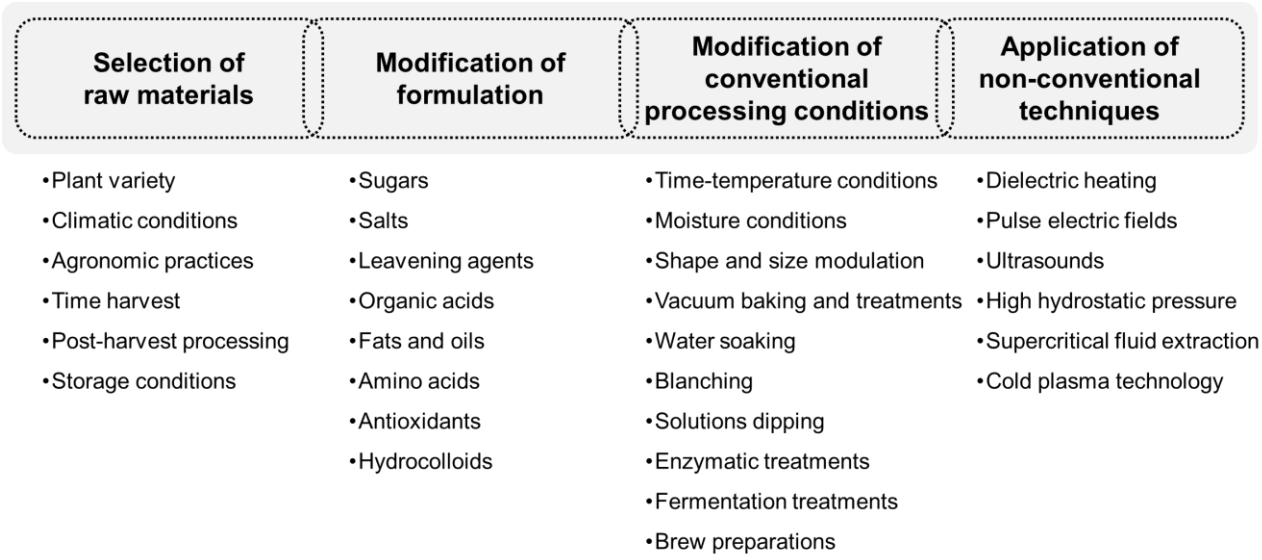


Figure 2.1 - Intervention strategies studied in the literature that can be applied alone or in combination to control acrylamide levels in food products.

The conventional technological approaches concerning AA reduction are related to changes in raw materials (e.g. selection of plants varieties with low reducing sugars and asparagine contents), selection of recipe or formulation (e.g. addition of specific ingredients affecting AA formation) as well as using pre-treatments (e.g. blanching or soaking) and modification in processing conditions (e.g. changing of time, temperature and pressure of heat treatment) (Anese, Suman, et al., 2009; Anese et al., 2013; Baskar & Aiswarya, 2018; Capuano & Fogliano, 2011; Maan et al., 2020; Nematollahi et al., 2021; Perera et al., 2021; Rannou et al., 2016; Rifai & Saleh, 2020; Šimko & Kolarič, 2019). In more recent years, several emerging and novel techniques including radiofrequency and microwaves heating, pulsed electric fields, ultrasounds, high hydrostatic pressure, supercritical fluids and cold plasma technologies have been used for AA reduction in

different food products (Baskar & Aiswarya, 2018; Maan et al., 2020; Nematollahi et al., 2021; Rannou et al., 2016).

Most of these strategies focus on achieving a low level of AA formation, while other methods propose to physically remove or reduce AA after its generation from the food products exploiting specific chemical and physical properties (Anese, Suman, et al., 2009; Anese et al., 2013; Nematollahi et al., 2021). Generally, preventing the formation of AA is the most widely applied method and can be obtained in two ways: reducing both or at least one of the AA precursors; and interfering with the Maillard reaction avoiding the subsequent generation of AA (Nematollahi et al., 2021; Zuo et al., 2015).

Each strategy approach may present limiting factors in its applicability depending on the type of product and the industrial context, i.e. feasibility and compatibility with the production process, impact on sensory and nutritional properties, compliance with safety regulations and production costs to be incurred. In some cases, limiting factors may be attributable to a lack of understanding of the influence of certain processes and variables (e.g. water activity and physical state of the product), as well as the close relationship between undesirable AA high levels and the development of desired sensory properties of foods. Indeed, since AA formation is concomitant with that of the colour, flavour, texture and some nutritional compounds of foods that have undergone cooking or roasting treatments, it is often very difficult to minimise AA without compromising the acceptability of the final product (Baskar & Aiswarya, 2018; Šimko & Kolarič, 2019). To ensure these challenges for mitigating the presence of AA without altering the desired nutritional and organoleptic properties inherent to the food product, more than one conventional or alternative strategy can be combined (Nematollahi et al., 2021; Perera et al., 2021).

2.2 Selection of raw materials

The amount of AA in processed products is strongly influenced by the raw material composition and quality which are affected by several factors such as plant variety and genetic, climatic conditions, agronomic practices, time of harvest, post-harvest processing and storage conditions.

The main issue to consider in mitigating AA through the selection of raw material is the concentrations of the main precursors, namely reducing sugars and free asparagine (Medeiros Vinci et al., 2012; Sarion et al., 2021; **Paper I**; **Paper V**). Regarding potato products, the reducing sugars content is generally considered the limiting factor for AA formation due to their lower concentration than asparagine (Raffan & Halford, 2019). On

the other hand, the AA generation in bakery products and roasted coffee is mostly owing to the low concentration of free asparagine (Bagdonaite et al., 2008; Seal et al., 2008). Therefore, the use of appropriate primary food ingredients with low concentrations of AA's precursors may help to reduce AA generation during the subsequent processing steps (Ciesarová, 2016; Nematollahi et al., 2021; Rifai & Saleh, 2020). For example, Medeiros Vinci et al. (2012) reported that large, long and oval potato cultivars contain high dry matter and lower amounts of reducing sugars, which makes them more favoured for the French fires' production, whereas moderate and oval tubers are mainly considered for chips. Recently, new cultivars such as Dakota Russet and Easton have been characterised, both of which showed significantly lower glucose and asparagine concentrations and consequently lower AA concentrations in the potato after processing (Sun et al., 2020). The choice of the variety is also important for cereal products, in this regard, Žilić et al. (2020) provided evidence of differences in the content of sugars and asparagine within 13 species of wheat, spelt, rye, barley, oat and maize. The resulting data indicated that hull-less oat, durum wheat and rye flours contained the highest content of free asparagine (859.8, 603.2 and 530.3 mg/kg, respectively), hence generating a higher amount of AA in biscuit products. Also, in roasted coffee products, the green coffee species plays a decisive role in the final amount of AA (**Paper I**). Among the two botanical species of greatest interest to the processing industry, a preference for Arabica over Robusta or a higher Arabica content in coffee blends have proven to be a decisive approach to achieving less AA formation during roasting (Bertuzzi et al., 2020). All these results showed that the chemical composition of raw materials, including reducing sugars and free amino acids, is controlled by specific genes present in certain varieties. Lower concentrations of reducing sugars and free asparagine would be achieved by altering genes effective in asparagine generation and degradation, carbohydrate metabolism and genes that could regulate reducing sugars (Y. Xu et al., 2014).

The seasonal climatic conditions variation may also influence asparagine and reduce sugar contents in the raw material (Medeiros Vinci et al., 2012; F. Xu et al., 2016). For instance, potato tubers grown in warm weather conditions (above 25-30 °C) and cold climates (below 8-12 °C) tend to show an increased sugars content of potato tubers and subsequently increased AA formation upon the heat treatment processing (e.g. frying and baking). Therefore, it is proposed that the optimum temperature for tuber growth ranges between 15 and 20 °C (Kumar et al., 2004). In addition, it has been established that AA generation in different bakery products is influenced by the presence of free asparagine

and reducing sugars that are associated not only with the cultivar but also with the crop's harvest season (Sarion et al., 2021). In fact, Claus et al. (2006), investigating the effects of climatic conditions during wheat and rye cultivation on asparagine contents in the grains harvested in 2003 and 2004, observed that their amounts were much lower in flours from the grains harvested in 2004 compared to 2003 harvests. The discrepancy in asparagine and protein contents between these flours was ascribed to different climatic conditions such as insolation and precipitations levels.

Regarding agronomic practices, soil fertilization has a central role in the generation of AA by having an impact on both asparagine and reducing sugar concentrations (Baskar & Aiswarya, 2018). For example, a decrease in nitrogen (N) fertilisation showed an increase in reducing sugars directly reducing the quality of the potato during frying; while an increase in N fertilisation in cereals crop resulted in high content of amino acids and protein causing an increase in AA levels in bread (Claus et al., 2006; De Wilde et al., 2006; Martinek et al., 2009; Sarion et al., 2021). With regard to sulphur (S) fertilisation, poor soils increase the level of asparagine, however, high values can alter the sensory properties of the flours, which is why a non-excessive but an appropriately balanced dose of S is recommended (Sarion et al., 2021). Other nutrients could be involved, such as phosphorus (P), potassium (K) and zinc (Zn), nevertheless, their incidence in AA formation in potato and bakery products is often contradictory probably because the effects of nutrition on the composition are variety-specific (Raffan & Halford, 2019; Rosen et al., 2018). It is clearly stated that an appropriate balance between the soil mineral composition, should be considered to obtain potato tubers less prone to AA development. However, it must be taking into account possible environmental impacts, legal fertilization limits and the need to prevent possible fungal and parasitic infections (De Wilde et al., 2006; Raffan & Halford, 2019).

Another factor that could contribute to higher AA levels is the rate of cultivar's maturity and ripeness that could affect the level of AA precursors. Unripe green coffee beans with lower quality (called defective beans) have significantly higher amounts of free asparagine than ripe ones; therefore, careful removal of these beans is recommended for the control of AA formation (Dias et al., 2012; Mazzafera, 1999). In addition, it was demonstrated that harvesting immature and thus smaller potato tubers are associated with higher reducing sugar amounts and a further increase in AA generation in the final fried potato products (De Wilde et al., 2006; Medeiros Vinci et al., 2012). Throughout tuber maturation, nutrients are transported from the leaves to the tuber and during vine

senescence prior to harvesting, a drop of sugar levels in the tuber is normally observed (Torres & Parreño, 2009).

In coffee products, AA levels are also related to the post-harvest processing conditions of the beans to provide a stable product for export, such as dry or wet processing (Knopp et al., 2006; Soares et al., 2014; **Paper I**). The amount of main reducing sugars, such as glucose and fructose, was less than 80% in wet-processed green coffee beans compared to dry-processed ones (Knopp et al., 2006). This difference in soluble solids can be explained by osmotic phenomena or anaerobic fermentation that occurs during wet post-harvest processing (Illy & Viani, 1995; Knopp et al., 2006). Despite these, Lantz et al. (2006) and Alves et al. (2010) did not find significant differences in AA concentration between dry- and wet-processed coffee bean samples. However, Lantz et al. (2006) did not show related data and Alves et al. (2010) compared AA results obtained only from 1 Arabica dry-processed sample versus 7 Arabica wet-processed ones (**Paper I**).

The formation of AA is also related to the storage time and temperature conditions of raw materials that can considerably affect AA precursors' content (Rannou et al., 2016). In this regard, storing the potatoes under low-temperatures (< 8 °C) conditions generally lead to a phenomenon called "low-temperature sweetening" which causes an increase in reducing sugar content and enhancement of the brown pigment during frying and hence higher amounts of AA (Chuda et al., 2003; De Wilde et al., 2006). However, storing potato tubers at a temperature above 8 °C can lead to senescent softening due to sprout growth and consequent degradation in tuber quality. This can be overcome by adopting an ideal temperature around 8-12 °C and spraying the harvest with chemical sprout suppressants (Halford et al., 2007). However, it has been established that the effect of storage conditions varies between cultivars and years (Medeiros Vinci et al., 2012; Sun et al., 2020). Proper storage conditions are also recommended for cereals, as it has been shown that sprouted grains should not be used for bakery products formulation. For example, bread prepared with sprouted grains had a 500% increment in AA compared to normal grains (Claus et al., 2006).

The selection of raw materials is of great importance as it directly influences the subsequent pathway mechanism of AA. Thus, there is a need for a careful choice of raw material quality in order to obtain food products containing as little AA as possible with satisfactory sensory and technological properties (Nematollahi et al., 2021; Rannou et al., 2016).

2.3 Modification of formulation

After careful selection of the main raw materials, changing any additional ingredients for food formulation is another important strategy to control AA formation in products that include this processing step, i.e. bakery products and possibly some formulated potato snack products but not roasted coffee (**Paper I**; **Paper V**). In detail, a change in the formulation can be achieved by replacing ingredients that contain AA precursors with others that are less likely to contribute to the Maillard reaction or by adding ingredients that can inhibit or slow down the AA production reaction (Anese, Suman, et al., 2009; Nematollahi et al., 2021). However, it must be taken into consideration that any formulation changes or ingredient addition aimed at reducing AA can significantly influence the overall quality of the final products. **Table 2.1** summarised some examples of ingredients studied for AA reduction and their probable inhibition mechanisms.

Table 2.1 - Examples of ingredients used for acrylamide reduction in different food products and possible reduction mechanisms.

Food product	Compound	Reduction	Mechanism	Reference
Sugars				
Gingerbread biscuits	Sucrose instead of ingredients rich in reducing sugars	95%	Decreasing the Maillard reaction rate due to lack of reactive carbonyl groups	(Amrein et al., 2004)
Low moisture model systems comparable to a shortcrust biscuit	Glucose instead of fructose	30%	Decreasing the Maillard reaction rate due to the high melting point of glucose	(Miśkiewicz et al., 2020)
Salts				
Cracker	NaCl	50%	n.r.	(Levine & Smith, 2005)
Biscuits	NaCl	20-25%*	Inhibition of the formation of the Schiff base between asparagine and reducing sugars	(Van Der Fels-Klerx et al., 2014)
Leavening agents				

Biscuits	No leavening agents instead of NH_4HCO	83-85%*	Activation of reducing sugars or formation of reactive sugar fragments by ammonia	(Kukurová et al., 2013)
Biscuits	$NaHCO_3$ instead of NH_4HCO_3	39-60%*	Less ammonia and addition of monovalent cations	(Sung & Chen, 2017)
Organic acids				
Biscuits	Tartaric acid	44%	Reduction of pH value and Maillard reaction rate	(Graf et al., 2006)
Biscuits	Tartaric acid	83%	Reduction of pH value and Maillard reaction rate	(Passos et al., 2018)
Fats and oils				
Fried potato chips	Corn oil instead of olive oil	66%	n.r.	(Becalski et al., 2003)
Short dough biscuits	Margarine	38%	Hamper the interaction between acrylamide precursors	(Anese et al., 2013)
Amino acids				
Biscuits	Amaranth protein isolate	26-89%*	Competitive interaction between asparagine and lysine/sulphur amino acids	(Salazar et al., 2012)
Biscuits	Cysteine and glycine	98%	Competitive interaction between asparagine and cysteine and glycine	(Zou et al., 2015)
Antioxidants				
Biscuits	Freeze-dried ginger powder	23%	Phenolic and the bioactive constituents alleviate protein glycation by trapping glucose thermal decomposition	(H. Yang et al., 2019)
Potato chips	Pomegranate peel nanoparticles extract	54%	Presence of pomegranate antioxidants	(Mekawi et al., 2019)
Hydrocolloids				

French fries	Pectin	48%	Reduction of the heat transfer impact	(Al-Asmar et al., 2018)
Biscuits	Pectin	67%	Reduction of pH value and Maillard reaction rate	(Passos et al., 2018)

n.r.: not reported by the authors of the reference.
* range covering different heat process conditions.

One of the most widely used ingredients in the formulation of various sweet food products are sugars. It has been widely reported that the right choice of type and amount of sugar is a possible strategy for limiting AA formation (Sarion et al., 2021; **Paper V**). Many studies have suggested that the replacement of reducing sugars (namely fructose and glucose) with sucrose (non-reducing sugar) is an effective approach to significantly reduce the AA content in bakery products. In gingerbread biscuits, a 95% decrease in AA content was reached by replacing honey, inverted sugar syrup and caramel colouring, with sucrose in an amount corresponding to the sum of glucose and fructose present in the previous list of ingredients (Amrein et al., 2004). The lack of reducing sugars, however, affected the development of the brown colour via the Maillard reaction. Because this is a desirable characteristic in most bakery products, substituting reducing sugars for AA reduction may be a viable option only for light-coloured products or bakery products used as ingredients (e.g. cake topping, dough enrichment). Comparing fructose and glucose reducing sugars, fructose exhibited a higher reactivity for AA formation as confirmed by the study of Miśkiewicz et al. (2020), who evaluated their effect in low moisture carbohydrate-asparagine model systems comparable to a shortcrust biscuit. The replacement of fructose with glucose or sucrose caused a decrease in the resulting AA content by 29.8% and 44.1%, respectively. These results were attributed to the low melting point temperature of the different sugars that have an impact on the degree of asparagine-to-AA conversion. In detail, sucrose, due to its high melting point temperature equal to 184 °C, is less reactive in Maillard reaction leading to a lower AA amount. On the other hand, fructose, having a very low melting point temperature (between 119 and 122 °C), is the most reactive leading to the highest formation of AA (**Paper V**).

Besides sugars, it has been reported that some salts (mono- or divalent cations) including sodium chloride (NaCl) as well as calcium chloride ($CaCl_2$) could decrease the presence of AA through the dehydration of various key Maillard reaction intermediate compounds, although conflicting results were found (Gökmen & Senyuva, 2007; Nematollahi et al., 2021; **Paper V**). In this respect, the addition of 1.3% w/w NaCl in a cracker system

decreased the AA level, nevertheless, a higher content of this salt promoted an AA increase (Levine & Smith, 2005). A possible method proposed to solve this problem could be the encapsulation of NaCl, useful to control the release of the reactants during the processing time (Fiore et al., 2012). Also in biscuits, the presence of a small amount of NaCl in the formulation lowered AA concentrations at a baking temperature of 180 °C and 190 °C but not at 200 °C, demonstrating that its effect is temperature-dependent (Van Der Fels-Klerx et al., 2014). Furthermore, in a model study comparing the effect of mono- (Na^+) and divalent- (Ca^{2+}) cations an equimolar amount in biscuits, it was found that the addition of Na^+ decreased the level of AA by approximately 70% while Ca^{2+} completely inhibited the formation (Gökmen & Şenyuva, 2007). However, when 1-2% w/w $CaCl_2$ was added to cracker and cake doughs with a reduction in AA of 60%, a failure to rise occurred, rendering the product unacceptable on a sensory level (Sadd et al., 2008). It is, therefore, necessary to assess the appropriate use of type and concentration of these salts in order not to adversely affect the appearance and sensorial proprieties of the final product.

Several studies have investigated the effects on AA levels of other salts commonly used as leavening agents in sweet bakery products, such as sodium bicarbonate ($NaHCO_3$), also called baking soda and ammonium bicarbonate (NH_4HCO_3) (Sarion et al., 2021; **Paper V**). It was extensively established that generally, any addition of leavening agent increased AA and that ammonium-based agents gave the highest levels while the replacement with $NaHCO_3$ could be a strategy to decrease the toxic compound (Amrein et al., 2004; Graf et al., 2006; Kukurová et al., 2013; Sadd et al., 2008; Sazesh & Goli, 2020; Sung & Chen, 2017). Although NH_4HCO_3 is the most widely used, it leads to an indirect increase in the formation of AA probably because it provides more reactive carbonyls originating from the reaction of ammonia with glucose and fructose. Glyoxal, methylglyoxal and many other formed R-dicarbonyls have been shown to react more rapidly with amino acids than glucose or fructose (Amrein et al., 2004). Nevertheless, it must be considered that different leavening powders in the formulation can significantly influence the final quality, especially the organoleptic characteristics. According to Graf et al. (2006), biscuits prepared without NH_4HCO_3 showed a lesser leavening volume as compared to the standard product formulated with a traditional baking agent composed of a mixture of NH_4HCO_3 and $NaHCO_3$. In addition, the addition of high doses of $NaHCO_3$ provides an alkaline taste, a yellowish surface colouration and an unpleasant taste (i.e.

soda bite) as well as an increase of sodium intake in the human diet (Canali et al., 2020; Cook et al., 2007; Kukurová et al., 2013).

Organic acids, usually added to food products to regulate acidity and improve flavour, have been tested in numerous studies in the literature for the control of AA formation (Nematollahi et al., 2021; **Paper V**). It is widely established that pH values can influence the Maillard reaction and the AA generation is maximum at pH 8. The addition of pH-lowering agents such as citric acid (e.g. from citrus fruits), lactic acid (derived from fermentation) and tartaric acid (e.g. from grapefruits) to the recipe can prevent the nucleophilic addition of asparagine with a carbonyl compound and the production of the corresponding Schiff base and thus the formation of AA (Nematollahi et al., 2021; Nguyen et al., 2017). For example, the addition of citric, tartaric, acetic and lactic acid as additives in the formulation of biscuits and other bakery products decrease the AA content (Amrein et al., 2004; Gökmen et al., 2007; Graf et al., 2006; Mestdagh et al., 2008; Mogol & Gökmen, 2016; Passos et al., 2018). Nevertheless, the amount of acid added is limited by the consequent modifications of the organoleptic and physic-chemical properties qualities that can occur (Passos et al., 2018). The addition of citric acid in gingerbread biscuits led to a product with a clearly acidic taste, not homogeneous colour surface and insufficient volume which limited their acceptability (Amrein et al., 2004; Gökmen et al., 2007). Furthermore, biscuits prepared with tartaric acid and examined by a sensory panel showed undesirable differences such as a harder, more brittle and sandy texture on the tongue and a more acidic taste than those prepared without any organic acid (Passos et al., 2018).

Fats and oils are added to the formulations of many food products to improve sensory and rheological qualities having also an impact on the AA content of the final product. In general, an increase in the fat content of biscuits reduces AA formation through a reduction in the interaction of AA precursors (Anese et al., 2013; **Paper V**). Moreover, the use of palm oil or margarine leads to a lower AA content than a monoglyceride-palm-oil-water gel, indicating that the incorporation of palm oil into the hydrogel form may modify the hindering effect of fat addition towards the formation of AA (Anese et al., 2013). However, even if the two fats had different chemical compositions and physical properties, no significant differences in AA formation were found between margarine and palm oil containing biscuits. Also, in fried potato products the type of oil used for frying can influence the levels of formed AA. Some studies indicated that palm oil relatively to rapeseed and sunflower oils or olive oil in comparison to corn oil generated higher AA

contents in French fries and potato chips (Becalski et al., 2003; Gertz & Klostermann, 2002), while other authors reported that oil type did not influence AA in the final product (Matthäus et al., 2004; Mestdagh et al., 2005; Williams, 2005). In addition, oil oxidation and hydrolysis products, which have been proposed as possible AA precursors, seem to be negligible in AA formation in fried potato products (Becalski et al., 2003; Mestdagh, De Meulenaer, et al., 2007; Mestdagh, Lachat, et al., 2007). On the other hand, the use of oxidized oil in biscuits formulation led to a huge increase in AA formation during baking (Arribas-Lorenzo et al., 2009). The type and amount of lipid influence the nutritional and sensory characteristics of the final product, thereby, further studies are needed to simultaneously evaluate the influence of fat/oil on both desired final characteristics and AA formation.

Another strategy to inhibit the formation of AA in several food products is the use of certain amino acids (e.g. with -SH and -NH_2 groups) and rich proteins ingredients (e.g. soy protein hydrolysates) that can compete with asparagine for the carbonyl group in Maillard reaction or that can react with the nucleophilic amino group of AA formed through Michael-type addition reaction, promoting its elimination (Casado et al., 2010; Medeiros Vinci et al., 2012; Mesías & Morales, 2016a). For instance, mixing of cysteine and glycine in the formulation process have been shown to effectively inhibit AA formation in asparagine/glucose model system as well as in real food system (Amrein et al., 2004; Zou et al., 2015). In a biscuit model, a significant decrease of about 97.8% in AA contents was observed using cysteine (0.36 g/100 g of dough) and glycine (0.2 g/100 g of dough) (Zou et al., 2015). Also, the addition of amaranth protein isolates significantly decreased the AA formation in the biscuits upon baking, from 89% (using a baking time of 7 min, which was the optimum baking time for the assayed biscuits) to 26% (with a baking time of 9 min). This result is due to the amino acid composition of amaranth proteins that are rich in lysine (4.8-6.4 g/100 g of protein) and sulfur amino acids (3.7-5.5 g/100 g of protein) leading to competitive phenomena between asparagine and amino acid residues and a reaction between amino and sulfhydryl groups with the AA formed (Salazar et al., 2012). However, even for these ingredients, it is necessary to evaluate possible modifications on the sensory proprieties of the final products. For example, the ready reaction between glycine and reducing sugars strongly increases the browning of the biscuit surface, as more melanoidins result, while the amino acid cysteine has an unpleasant taste and odour presumably caused by S-containing decomposition products (Amrein et al., 2004). It must also be considered that the use of amino acids as pure compounds in foods is not easy

because their use is strictly regulated in terms of quantity, nature and application depending on the legislation of each country (Rannou et al., 2016).

Several antioxidant ingredients, including also various plant extracts (rich in phenolic and antioxidant compounds), could be effective in controlling AA formation (Jin et al., 2013; Mekawi et al., 2019; C. Xu et al., 2015; H. Yang et al., 2019). Some antioxidants were claimed to reduce AA formation, while others did not show any effect or showed an enhancing effect according to the type of antioxidants compounds, their concentration and purity (Özge Ç Açar & Gökmen, 2009; Constantinou & Koutsidis, 2016; Hedegaard et al., 2008). The positive effect of antioxidant compounds could be related to their reaction with AA precursors or intermediates, which may inhibit the overall rate of the Maillard reaction. In particular, these ingredients could control AA formation in three ways: by trapping of carbonyls, reduction of sugar degradation through Maillard reaction processes and radical scavenging activity (AL-Ansi et al., 2019; Demirok & Kolsarici, 2014). For example, ground freeze-dried ginger added in different amounts 1-7% w/w in biscuit formulation was able to significantly reduce the AA content with dose-dependent relation. This result was attributed to the phenol hydroxyl group of gingerol and the bioactive constituents that alleviate protein glycation by trapping glucose thermal decomposition product called methylglyoxal, which might affect the inhibition of AA formation (H. Yang et al., 2019). On the other hand, grape seed extract added to baked products did not have any effect on AA formation (Özge Ç Açar & Gökmen, 2009). This might be due to specific roles played by factors arising from different types of food ingredients (Maan et al., 2020). Another study discovered that lyophilized pomegranate peel nanoparticles extract into sunflower oil (1000 mg/kg) could be favourably used as an antioxidant for AA reduction in potato chips during deep frying. The relatively low levels of AA in antioxidant-enriched oils were attributed to the protective action of antioxidants against thermo-oxidative degradation (Mekawi et al., 2019). However, the effect on the sensory characteristics of the finished fried potato was not determined. As antioxidants are commonly added by plant or spice extracts, some work in the literature has found an impact of these ingredients on sensory characteristics. For example, a sensorial panel evaluation results showed that the colour, texture and flavour of biscuits processed with either bamboo leaves (0.2 g/kg) or vitamin E (0.1 g/kg) did not differ significantly from control biscuits. Nevertheless, other ingredients such as polyphenols powders from virgin-olive oil mill wastewater are characterized by bitterness and astringency, especially when added at high concentrations (Troise et al., 2020).

Other minor compounds such as hydrocolloids including Arabic gum, chitosan, pectin and alginic acid were investigated for AA reduction in foods. The inhibition mechanism is based on the molecular movements to make interactions between hydrocolloids and the precursors of AA (Mousa, 2019). Some hydrocolloids are already widely used in food industries to modify the functional properties of aqueous food systems, reduce the fat uptake when used as coating agents and to improve the technological characteristics (García et al., 2004; Medeiros Vinci et al., 2012). For instance, coating treatments with alginic acid and pectin decreased the amount of AA in French fries (Al-Asmar et al., 2018; Zeng et al., 2010). This was ascribed to the formation of surface coatings that may impact the heat transfer between the frying oil and the potato strips, consequently, alter the AA formation (Zeng et al., 2010). Other hydrocolloids such as pectin and pectate also could apply as an AA mitigation strategy in bakery products because of the decrease in pH (Passos et al., 2018). In contrast, carob gum, carrageenan, hydroxypropyl distarch phosphate, xanthan gum and chitosan enhanced the quantity of AA in fried French fries and biscuits systems (Mogol & Gökmen, 2016; Zeng et al., 2010).

Although a large variety of ingredients have been investigated with some proven effectiveness against AA formation, their real industrial applications will strictly depend on the type of the product and their effectiveness against AA formation without affecting the final product quality and safety (Maan et al., 2020).

2.4 Modification of conventional processing conditions

The main processing parameters, including temperature, time, moisture content, size and pH, play a critical role in AA formation affecting the Maillard reaction behaviour. Thus, the proper change in these factors could be considered an efficient AA mitigation strategy through heat treatments. Some examples of conventional processing conditions proposed for AA reduction and their probable inhibition mechanisms are summarised in **Table 2.2**.

Some investigations outline an important relationship between cooking time and temperature, demonstrating that an appropriate choice of these process parameters can be an effective way to minimise AA during the heat treatment of different food products (Anese, Suman, et al., 2009). This can be achieved in bakery products, for example, by applying prolonged heating at lower temperatures or by optimising the thermal profile adopting higher temperatures in the initial stages of heating, when the moisture content

is high, followed by lower temperatures in the later stages, when the water content is low (Haase et al., 2012; Romani et al., 2008; Vass et al., 2004).

Table 2.2 - Examples of modification of conventional processing conditions studied for acrylamide reduction in different food products.

Food product	Strategy	Reduction	Mechanism	Reference
Time and temperature conditions				
Roasted coffee	High heating time and temperature	90%	Degradation of acrylamide formed in early stages	(Şenyuva & Gökmen, 2005)
Biscuits	Low baking temperature and long-time	68%	Reduction of Maillard reaction rate	(Haase et al., 2012)
Moisture conditions				
Roasted coffee	Steam roasting	10%	n.r.	(Theurillat et al., 2006)
Biscuits	Steam baking instead of forced convection baking	32-46%*	Reduction of surface temperature rise	(Isleroglu et al., 2012)
Shape and size modulation				
French fries	Strip samples of 14×14 mm instead of 8×8 mm	37***	Low surface-to-volume ratio	(Matthäus et al., 2004)
Biscuits	Thick sample of 10 mm instead of 1 mm, baked at 180 °C for 15 min	100%	Change in temperature distribution in different biscuit areas	(Özge Ç Açar & Gökmen, 2009)
Vacuum baking and treatments				
Biscuits	Vacuum baking	72-76%*	Differences in drying rate of biscuits	(Mogol & Gökmen, 2014)
Potato crips	Only vacuum baking and combined conventional-vacuum baking	98% and 95%	Lower temperatures due to the lower boiling point of water	(Akkurt et al., 2021)
Water soaking treatments				

French fries	Soaking in distilled water for 60 min	25%	Leaching of acrylamide precursors from the surface layer of potato cuttings	(Jung et al., 2003)
Potato chips	Soaking in distilled water for 90 min	32%	Leaching of acrylamide precursors from the surface layer of potato slices	(Pedreschi et al., 2004)
Blanching treatments				
French fries	Low temperature and long-time blanching treatments	59%	Increased sugar release in the soaking water	(Pedreschi et al., 2007)
Potato chips	Blanching 3 min at 80 °C	51-73%**	Release of acrylamide precursors in the soaking water	(Viklund et al., 2010)
Solutions dipping treatments				
Potato chips	Acetic acid dipping (at 20 °C for 60 min)	90%	Reduction of pH value and Maillard reaction rate	(Kita et al., 2004)
Potato chips	NaCl dipping (at 25 °C for 5 min)	62%	Inhibition of the reaction between asparagine and reducing sugars and facilitated diffusion of NaCl into the tissue	(Pedreschi et al., 2010)
Enzymatic treatments				
French fries	Asparaginase solution at 40 °C for 20 min	60%	Reduction of asparagine content	(Pedreschi et al., 2008)
Roasted coffee	Asparaginase (80%)	74%	Reduction of asparagine content	(Hendriksen, 2013)
Fermentation treatments				
Instant coffee	Beverage yeast fermentation	70%	Acrylamide degradation by yeast metabolism	(Akıllıoglu & Gökmen, 2014)
Bread	Dough yeast fermentation	55-61%**	Reduction of asparagine content by yeast metabolism	(S. Wang et al., 2017)
Brew preparations				
Coffee drink	Espresso extraction	75%	Minor acrylamide extraction efficacy due to	(Lantz et al., 2006)

			highest coffee-to-water ratio	
Coffee drink	Espresso extraction	71%	Different extraction method	(Soares et al., 2006)

n.r.: not reported by the authors of the reference.
* range covering different heat process conditions.
** range covering different raw material varieties.
*** percentage calculated from the graph of the reference manuscript.

However, although it is a promising strategy for reducing AA, a modification of frying temperature might not always be feasible, as the Maillard reaction is also responsible for the formation of colour and aroma components and an increase in frying time could enhance fat uptake (Foot et al., 2007; Romani et al., 2008). Moreover, in general, it must be specified that at the industrial level long heat treatment times reduce the efficiency and time of the production line (Anese et al., 2013). Another mitigation strategy is to apply high temperatures combined with long processing times that lead to low AA levels due to physically elimination/degradation reactions (**Paper I**). However, such intense heat treatments are responsible for great development and advancement of non-enzymatic browning reactions and, consequently, of important changes in sensory attributes and nutritional quality. Although these changes are not desired for most foods, as the product may be unacceptable for consumption, they are desired for other types of food, such as coffee products (Anese, Suman, et al., 2009; Konings et al., 2007). In this regard, Şenyuva and Gökmen (2005) showed an increase in the AA content of coffee, reaching a maximum level after 10 and 5 min of heating at 200 and 225 °C respectively, and a subsequent decrease in AA as the heating time was extended. These results were also confirmed during an industrial coffee roasting process by the recent study of Bertuzzi et al. (2020). Until now, no research work reported a possible mechanism of AA evaporation or degradation during extended coffee roasting. Only Pastoriza et al. (2012) proposed a possible pathway studied on a low-moisture model system concluding that the decrease of AA during roasting is due to its chemical interaction with coffee melanoidins, whose concentration have a direct effect. In order to explain these results, the authors hypothesized that nucleophilic amino groups of amino acids from the proteinaceous backbone of melanoidins react via the Michael addition reaction with AA. However, further studies are necessary to identify the specific mechanisms of this reaction and to clarify if the degradation of AA leads to the possible formation of other toxic compounds, which may harm human health. However, until now, very few research works attempted to identify a potential mechanism of AA evaporation or degradation during prolonged

roasting. Recently, Badoud et al. (2020) investigated the fate of AA during roasting and brew preparation using ^{14}C- and ^{13}C-labeled AA. The results highlighted the complexity of the reactions involved in coffee roasting and indicated that while about 25% of AA was lost by volatilization, the remaining 75% was detectable in the final products, but only 50% was in free soluble form.

Closely related to heating temperature and time, the moisture level also plays a crucial role in the formation of AA in foods. As is well known, both the total amount and activity of water present in foods greatly influences the reactivity of chemical constituents, including AA precursors and reactions. It has been found that AA formation is enhanced with initial water activity in the range of 0.4-0.8 and a moisture content < 5% (De Vleeschouwer et al., 2007; Elmore et al., 2005). In addition, the content and activity of water can change during the heating process, thus affecting the rate of AA formation. It has been shown that as long as the water does not evaporate (temperature does not exceed 100 °C), no formation of AA is detected (Anese, Quarta, et al., 2009; Anese, Suman, et al., 2009; Bråthen & Knutsen, 2005). Conversely, the lower the moisture in the product, the more AA is formed at the same temperature (Anese, Quarta, et al., 2009; Anese, Suman, et al., 2009; Elmore et al., 2005). High relative air moisture during baking has been shown to be effective in reducing AA levels in bakery products (Ahrné et al., 2007; Isleroglu et al., 2012; Konings et al., 2007; J. Li et al., 2016). This can be achieved not only by reducing the temperature but also by injecting steam during baking. In respect, Isleroglu et al. (2012) showed that steam-assisted oven did not raise the biscuit surface temperature above the values that were observed at the convectional ovens (forced or natural convection) leading to less AA formation, especially at low baking temperature (165 and 180 °C). However, this solution is suitable for foods with relatively high final residual moisture, whereas many heat-treated food products need to reach a lower moisture content for their acceptability (Anese, Suman, et al., 2009). For example, Theurillat et al. (2006) evaluated in Arabica and Robusta coffee two different steam treatments applied during roasting, using respectively an autoclave and a rotating roaster equipped with steam injection. The results showed that the steam treatments performed did not have a significant impact on the final AA content, furthermore, the steam treatments resulted in a worse sensory profile of the coffee, which was more acidic, less roasted and bitter than conventionally roasted coffee making this solution not applicable. As not only the temperature and heating time but also the heat transfer mechanism, are considered essential process parameters affecting AA generation, vacuum treatments

can be used to manage AA levels in some potato products, baked goods and coffee. Recently, Akkurt et al. (2021) studied the effect of low pressure (10 mbar) during baking in AA levels in baked reformulated potato chips. Compared to conventional oven baking, vacuum baking led to a decrease in AA formation in baked potato chips up to 98% and the combination of the two baking methods let to decrease up to 95% thanks to the decrease of the boiling point of water and consequently reduce thermal load (heating time and temperature). The vacuum-baked and combined baked potato chips were found to be lighter in colour; nevertheless, the sensory analysis showed that vacuum-baked samples were more appreciated than the others in texture and overall acceptability. A considerable reduction in AA content was also found by Granda and Moreira (2005), Palazoğlu et al. (2015) and Yıldız et al. (2016) who tested the use of vacuum frying of potatoes. Similar results for AA reduction through vacuum-combined baking have also been reported by Mogol and Gökmen (2014) in biscuits. However, in this type of product, the main disadvantage of this technique is the probable simultaneous lack of typical and desired surface colour making this mitigation technique feasible only for products with a coating or the addition of coloured ingredients (Mogol & Gökmen, 2014). The possibility of low-pressure heating for AA control was also evaluated in roasted coffee (Anese et al., 2014). Under reduced pressure to a medium degree roasting, it was possible to obtain AA levels 50% lower than samples obtained under conventional and combined conditions without affecting the colour and sensorial characteristics. Probably, the vacuum conditions generated inside the roaster exerted a stripping effect, preventing AA from being accumulated. Nevertheless, this AA mitigation strategy could be of limited interest since the coffee roasted at a medium degree is consumed almost only in the American and Northern European markets (**Paper I**). Vacuum technology can also be applied as a post-heating treatment because, based on its low molecular weight, AA can be removed from the food together with steam. Pressure and temperature conditions of 6.67 Pa and 60 °C have successfully been applied to remove AA from biscuits (−43%) and potato chips (−18%) previously hydrated at a high-water activity of 0.83 (Anese et al., 2010). However, this research did not evaluate the effects of the post-treatment on the sensory properties. In principle, this technology can be applied to any finished product without altering recipes and process parameters. Nevertheless, vacuum frying is a quite expensive process with smaller capacities as compared to the standard heating method and its impact on overall manufacturing practices and food quality have not yet been clearly established (Anese et al., 2013).

In general, AA is formed on the surface layer of the products therefore, their size and shape before the cooking process may exert an influence on the final amount of AA. Concerning fried potatoes, the thinner and smaller the size of the cut portions, the higher the concentration of AA that forms on them (Matthäus et al., 2004). Other factors to consider during frying include the contact surface and the volume of potato samples: a large contact surface leads to much greater amounts of AA because more precursors are exposed to high temperatures. On the other hand, the more the amount of sample added into the oil the greater the temperature drop and the longer the frying time required to reach the initial temperature. Therefore, AA formation diminishes, but the frying quality is degraded. In addition, since the peripheral region of tubers has a higher content of reducing sugars, fine cuts from the outer area of tubers tend to overheat during frying. Thus, removal of these fine cuts may contribute to a reduction in AA (Foot et al., 2007). The influence of dough thickness in 1 mm (crust model) and 10 mm thick biscuits on the AA content in the crust of the finished product was also investigated monitoring the change of temperature in different locations of the sample. During baking, the temperature values reached were similar in the different areas of the 1 mm thick biscuit, reaching a baking temperature of 180 °C in approximately 12 min, whereas, at the same oven temperature, the dough did not exceed 100 °C after 30 min in the 10 mm thick sample. Consequently, in 1 mm thick biscuits, AA formation increased rapidly within 8 min and then decreased exponentially due to its thermal degradation. In contrast, under the same firing conditions, no AA was detected in the 10 mm thick sample within 15 min as the moisture content was still above 10% and AA maximum level was reached only after 30 min (Özge Ç Açar & Gökmen, 2009). Controlling size and shape is a very simple strategy for controlling AA; however, it is not always sufficient and feasible depending on the product.

In addition to changes in heat conditions and methods, other integrated conventional treatments before food heat processing could affect AA generation considerably. Among them, a simple measure of pre-soaking in water can promote the mass transfer phenomena of AA precursors before the heat-treatment process consequently reducing the formation of AA. This strategy has been successfully used in potato products, washing raw French fries in distilled water-induced 24.9% reduction of AA formation (Jung et al., 2003). Similarly, soaking potato slices in distilled water for up to 90 min will reduce sugar and AA contents by about 30% (Pedreschi et al., 2004). That seemed to be merely due to the leaching out of free asparagine and reducing sugars from the surface layer of potato

cuts into the distilled water during dipping (Jung et al., 2003; Medeiros Vinci et al., 2012; Pedreschi et al., 2004). Moreover, this pre-treatment allowed to lower frying times to produce an acceptable final product (Pedreschi et al., 2004). This technique is promising for the control of AA, but it is not always easily applicable on an industrial scale due to the time required for treatment.

To reduce and improve soaking treatments, this step can also perform in hot water (blanching treatments) because enhance the extraction of sugars from the raw food matrix (Maan et al., 2020; Pedreschi et al., 2007). Blanching is an established unit operation practised in the fried potato' food industry for limiting the absorption of oil, improving the texture, colour retention and enzyme inactivation (Baskar & Aiswarya, 2018; Maan et al., 2020; Michalak et al., 2020). A reduction ranging between 51% and 73% in AA levels was reported in three long-term stored potato clones' chips; possibly due to a decline in the diffusion of precursors to the surface of slices (Viklund et al., 2010). In addition, it has been demonstrated that as the blanching temperature and duration increase, more glucose and asparagine are being leached out leading to French fries with lower AA levels (Pedreschi et al., 2007). However, the conditions of soaking and blanching can be varied within certain limits, it is necessary to modulate them to avoid problems such as loss of desired texture properties and increased oil absorption causing several nutritional drawbacks (Mariotti et al., 2015; Medeiros Vinci et al., 2012).

A similar powerful pre-treatment to decrease AA generation is to dip unprocessed foods in solutions containing inhibitor compounds that could affect AA formation, such as organic acids, amino acids and salts (Abboudi et al., 2016; Maan et al., 2020; Meghavarnam & Janakiraman, 2018; Rannou et al., 2016; Zou et al., 2015). One example is the dipping in acid solution (e.g. citric and acetic acid), usually applied for lowering the pH of the food matrix, which converts free non-protonated amines to protonated amines, which could block the formation of AA (Kita et al., 2004; Rydberg et al., 2003). However, a drawback of this treatment is that it can impart undesirable acidic flavours and aromas to the finished product (Friedman & Levin, 2008). Another study by Pedreschi et al. (2010) evaluated the combination of blanching treatments and NaCl solution dipping over the AA formation in potato chips. Blanching followed by the immersion of potato slices in NaCl solution (1 g/100 g) was effective in reducing AA content of about 62%; almost half of this percentage (27%) could be attributed to the effect of NaCl and 35% to the effect of the slight heating treatment during salt immersion step (25 °C for 5 min). Blanching seems to

make the NaCl diffusion in potato tissue easier leading to a significant AA reduction in the potato slices after frying (Pedreschi et al., 2010).

Dipping treatments can be used to apply an enzymatic L-asparaginase treatment, one of the most promising and extensively used AA mitigation techniques (Banchero et al., 2013; Pedreschi et al., 2011). In particular, the application of L-asparaginase, under a specific time, temperature and pH conditions, catalyses the specific hydrolysis of asparagine into aspartic acid and ammonia before heat treatment is applied and the Maillard reaction takes place (Ciesarová, 2016; Krishnakumar & Visvanathan, 2014; Medeiros Vinci et al., 2012; Meghavarnam & Janakiraman, 2018; Rannou et al., 2016; Zuo et al., 2015). Asparaginases can be derived from various sources including plants, bacteria, yeast, fungi, actinomycetes, algae and serum of some rodents (Munir et al., 2019; Sharma & Mishra, 2017). Commercial forms of this enzyme already established as safe by FAO/WHO and FDA and already used for industrial applications are from the fungi *Aspergillus oryzae* and *Aspergillus niger* (Corrêa et al., 2021; Jia et al., 2021; Muneer et al., 2020; F. Xu et al., 2016). For this purpose, Hendriksen (2013) evaluated the effect of commercial asparaginase produced by *A. oryzae* on raw green coffee obtaining a decrease of 55-74% of AA after roasting. In addition to this application, various patents suggested the use of L-asparaginase combined with various techniques aimed at improving the contact between enzyme and the food substrate, overcoming the incomplete hydrolysis of asparagine. For example, as the green coffee bean is a very dense, hardly permeable matrix, it was suggested to pre-treat it by drying, hydrating, grinding, steaming and soaking in bath water, eventually in combination with reduced or increased pressure with the aim to create a driving force for the enzyme solution to easily migrate into the coffee beans (Dria et al., 2007; Lynglev & Schoesler, 2016; Navarini et al., 2014). Following this concept, a study has demonstrated that a combination of L-asparaginase dipping and blanching at 40 °C for 20 min as a pre-treatment stage, reduced AA formation during French fires frying by 60% when compared with blanched strips without the enzyme treatment (Pedreschi et al., 2008). Asparaginase treatments, during the leavening process, was promising also to reduce AA in leavened sweet bread (Meghavarnam & Janakiraman, 2018). Before baking, the raw dough was natural fermented and treated with asparaginase from *Fusarium culmorum* and left to rest at 30 °C for 90 min, then stirred and left to rest again for 25 min, resulting in an 86% reduction in AA. Although it has been generally stated that this AA mitigation strategy is very powerful because it does not adversely affect aspects of the final product, due to the

multiplicity and complexity of variables affecting enzyme activity, it requires optimisation as well as evaluation of stability and recyclability to consolidate its application in the food industry (Jia et al., 2021; Rifai & Saleh, 2020).

A further intervention considered an interesting strategy to minimise AA in various foods due to the fact it has limited impacts on the sensory properties of the final product, is the precursors' reduction through fermentation treatments processes using some microorganisms (Albedwawi et al., 2021; **Paper I**; **Paper V**). Fermentation is an important process in the preparation of leavened savoury bakery products and can control the kinetics of AA formation by eliminating reducing sugar and amino acids and lowering the pH of the matrix due to the microbial metabolism (Albedwawi et al., 2021; Maan et al., 2020). In fact, extending the biological fermentation time and allowing the enzymatic kit of the yeasts used to consume asparagine is a powerful strategy to reduce the AA formation in bakery products (Anese, Suman, et al., 2009; Sadd et al., 2008). In a bread product, depending on the type of flour used, fermentation by different yeasts resulted in a reduction of asparagine in the dough and AA in the baked bread in the order of 40-60% and 55-61% respectively (S. Wang et al., 2017). In addition, in potato products, the lactic acid fermentation treatments before frying have shown to be effective in reducing AA (Albedwawi et al., 2021; Baardseth et al., 2006; Blom et al., 2009). A greater reduction in AA of fried potato can be achieved by combining lactic acid fermentation with blanching in water without affecting the desired sensory propriety (i.e. browning, flavour, acidity and freshness) and consumer preference (Anese, Bortolomeazzi, et al., 2009). Fermentation was also successfully applied to remove AA from instant coffee (soluble coffee) using the yeast *Saccharomyces cerevisiae*. This fermentation process can adapt to the normal instant coffee production line reaching and, after 48 hours, allowed to reach a reduction of 70% of AA because of the metabolic activity of the yeast (Akıllıoglu & Gökmen, 2014). It should be noted that some industrial-scale investigations are needed to evaluate important aspects concerning the concentration of microorganisms to be used and their fermentative power during treatment, as well as the identification of appropriate application conditions (time and temperature) to make the most of their activity. In addition, identifying ways to keep the bacteria viable for a few days would allow the reuse of the micro-organisms and ultimately reduce costs (Anese, Suman, et al., 2009).

About the coffee drink, the method of preparing the brew can affect the amount of AA present. As AA is a highly water-soluble compound, during the brewing process it may be more or less extracted in the coffee drink depending on the extraction conditions

(Andrzejewski et al., 2004; **Paper I**). All brewing parameters, such as coffee grinding degree, coffee-to-water ratio, pressure, extraction temperature and time according to geographic, local traditions and social context, may significantly affect the AA level in the final coffee beverage (Cordoba et al., 2019; **Paper I**). For example, Lantz et al. (2006) compared AA in coffee beverages obtained by different brewing systems (i.e. Horeca pour-over system, household drip-filter, plunger pot, fresh-brew filter of vending machine and espresso) and reported that the use of espresso method leads to the lower AA extraction (75%). The minor extraction efficacy in coffee espresso brew was attributed to the highest coffee-to-water ratio (146.0 g/L) and to the shortest extraction time. In agreement, a higher AA means level in the soluble coffee brew (72.4 µg/L) compared to espresso one (21.0 µg/L) was reported by Soares et al. (2006). Following this positive result of espresso brewing, the influence of espresso coffee volume on the AA was also investigated, demonstrating that a long espresso (70 mL) leads to the highest AA extractability of 98-99% than shorter espresso coffees (20-50 mL) due to the longest contact time between coffee powder and hot water (Alves et al., 2010).

Careful choice of processing variables and application of additional conventional processing steps can be used as an effective tool to reduce AA in several thermally processed foods. However, depending on the foodstuffs, each process modification adapted must be evaluated and optimized for many different constraints (nutritional, sensorial, economic and technological) to obtain the desired final product characteristics (Maan et al., 2020).

2.5 Application of unconventional process techniques

In recent years several new processing techniques, alone or in combination with conventional ones, have been proposed as potential tools to minimise the formation of AA in a large variety of food products (Abboudi et al., 2016; De Vleeschouwer et al., 2010). Some examples of unconventional processing conditions that can help to reduce AA levels minimizing even the slightest alterations to the final nutritional and sensorial proprieties are reported in **Table 2.3**.

Some unconventional heat-transfer methods, in combination with conventional baking or not, are suggested to reduce the thermal input during the processing of the products. An alternative is dielectric heating (i.e. radio-frequency and microwaves), a novel technology that allows rapid and uniform heating because heat is generated within the product due

to the frictional interactions of polar dielectric molecules rotating in response to an externally applied alternating current electric field (Mogol, 2015). As water is the target molecule of dielectric heating, heat is generated at any site of the food where water is present (Anese et al., 2013).

Table 2.3 - Examples of application of unconventional processing conditions used for acrylamide reduction in different food products.

Food product	Compound	Reduction	Mechanism	Reference
Dielectric heating				
Biscuits	Post-baking with radio-frequency heating	50%	Removal of water without overheating the food surface	(Palazoğlu et al., 2012)
French fries	Pre-treatment with microwaves heating	60%	Reduction in heating time for subsequent conventional frying	(Erdoğdu et al., 2007)
Pulsed electric fields (PEF)				
Potato chips	PEF instead of blanching raw potato slices pre-treatment	30%	Great leaching of acrylamide precursors	(Genovese et al., 2019)
Potato chips	PEF pre-treatments to whole raw potato tubers	17%	Achieving a smooth cut with a consequent reduction in surface starch particles	(Ostermeier et al., 2021)
Ultrasounds (US)				
Potato sticks	Pre-treatment with ultrasounds soaking	44%	Reduction of reducing sugar contents	(Antunes-Rohling et al., 2018)
Potato chips	Pre-treatment with ultrasounds soaking	95%	Reduction of reducing sugar contents	(Pedreschi et al., 2021)
High hydrostatic pressure (HHP)				
Asparagine-glucose model system	High pressures (100-300 MPa for 60 min at 120 °C)	70-86%*	Delay of the initial phase of the Maillard reaction	(Kobayashi et al., 2019)
Potato sticks	High pressures (100-400 MPa for 5 min) before	26-47%**	Induction of asparaginase	(Dourado et al., 2020)

	immersing raw potato sticks in asparaginase solution		infusion due to structural changes caused in potato tissue and possibly changes in the enzyme functionality	
Supercritical fluids technology (SFT)				
Roasted coffee	Supercritical CO_2 extraction after the roasting process	79%	Solubilization of formed acrylamide in the supercritical fluid	(Banchero et al., 2013)
Cold plasma technology				
Roasted coffee	Corona discharge plasma jet treatment after the roasting process	30%	Degradation of formed acrylamide	(Lee et al., 2020)

* range covering different studied conditions.
** range covering different treatments conditions.

Thus, radio-frequency (RF) is considered a feasible post-treatment drying method to lead a rapid decline in the product's water content keeping low the AA concentration in bakery products (Anese et al., 2008, 2013; Kocadağlı et al., 2012; Mogol, 2015; Palazoğlu et al., 2012; Rannou et al., 2016; Sarion et al., 2021). As is well known, in the final stages of conventional cooking, the surface moisture of the product is very low, requiring a considerable time to cook the centre due to low thermal conductivity and thus causing a large amount of AA content generated by the Maillard reaction (Fellows, 2000). Following this concept, Palazoğlu et al. (2012) observed a noticeable reduction in AA by up to 50% during conventional biscuit baking (205 °C for 8 min) combined with a short RF heating (45 s) as a post-drying step compared to control samples. This was attributed to the use of a lower temperature in this emerging technology, removing water without overheating the surface of the food sample. However, the use of RF to finish the baking of biscuits leads also to lower surface browning development and present a slight uncooked flavour (Kocadağlı et al., 2012; Palazoğlu et al., 2012). To overcome the consumers' acceptability, a strategy is to introduce preformed Maillard reaction products (e.g. melanoidins), characterised by a typical colour and flavour, in the biscuit dough before proceeding with baking (Kocadağlı et al., 2012). The viable treatment of post-drying RF heating has also been proposed to mitigate AA formation in potato chips (Koklamaz et

al., 2014). In detail, this post-drying approach resulted in decreasing the AA content by up to 32% in the final fried potato, while also maintaining the desired final moisture content and quality attributes compared to conventional chips fried in oil at high temperatures (Koklamaz et al., 2014). Microwaves (MW) heating can also be an alternative method to reduce AA formation in bakery and potato products (Michalak et al., 2020). For example, in biscuit products MW baking (at 700 W for 90 s) reduces the AA level by 10% compared to those conventionally baked at 190 °C for 10 min (AL-Ansi et al., 2019). The beneficial effect of MW was also confirmed by the application of an MW pre-cooking step (30 s at 850 W) in French fries, fried at 190 °C for 1.5 min, reaching an AA reduction of 60%. These results were expected, since the time of frying employed to obtain the same degree of cooking decreased with the MW pre-treatment due to the change in potato structure (Erdoğdu et al., 2007). Despite the excellent results in AA reduction, MW treatment also led to several disadvantages on a sensory level such as insufficient brown colour on the surface, poor crust formation, irregular upper surface with some bulges (AL-Ansi et al., 2019; Michalak et al., 2020). In addition, some authors, in model systems and different foodstuffs, have suggested that more AA can be formed with MW heating than with conventional heating methods (Juodeikiene et al., 2018; Michalak et al., 2011, 2020). As an example, Michalak et al. (2011) found that MW heating (at 700 W for 10 min) of frozen pre-prepared potato products (e.g. French fries, cubes, wedges, noisettes, pancakes) led to higher levels of AA in the final product than any other home-cooking method such as pan-frying, deep-frying and roasting. Probably, MW heating provides a favourable medium for the kinetics of AA formation as a result of rapidly reaching high temperatures inside a low thermal conductivity product (Michalak et al., 2011). It seems that contradictory results on the effect of MW heating on the AA formation in foods may be mainly due to differences in MW parameters (power, heating time) used, method of application (combined or not with conventional systems) as well as the chemical composition and type of heated food, including water activity levels (Michalak et al., 2020). In summary, the use of specific dielectric heating conditions alone or in combination with conventional heating to mitigate AA presents numerous advantages. Indeed, it does not require changes in food recipes and can be applied to simultaneously mitigate AA and furan compounds. Other advantages include energy savings, heating independent of the product thermal conductivity and reduced production floor space requirements. In addition, this technology can be easily adapted to both automated production batch and continuous flow processing (Anese et al., 2013). However, an in-

depth study is needed in the MW technique to find a compromise between the amount of AA generated and the sensory characteristics of the dielectric-heated food.

The treatments such as pulsed electric fields (PEF), ultrasounds (US) and high hydrostatic pressure (HHP) treatments are considered non-thermal processes that support removing asparagine and reducing sugars from the matrix of raw materials, resulting in AA formation inhibition in different food products (Abboudi et al., 2016; Anese et al., 2013; Rannou et al., 2016). To date, these alternative technologies have been applied in fried potato products.

In terms of PEF, this technique is based on the application of short pulses (in the range of µs to ms) of high electric fields to foods leading to the permanent or temporary disintegration of the cell membrane, depending on the treatment intensity (Ignat et al., 2015; Jaeger et al., 2010). Genovese et al. (2019) compared the PEF application with conventional blanching as a pre-treatments of raw potato slices before frying to decrease the potato chips' AA level. Their results proved that the PEF, as a non-thermal treatment, leached significantly higher AA precursors than the thermal blanching process, which led to more AA reduction in the final product (Genovese et al., 2019). In agreement, a reduction of 17% AA was reached by applying PEF pre-treatment of whole potato tubers before cutting and without the following washing (Ostermeier et al., 2021). A possible explanation for why a reduction in AA was achieved when PEF was applied in whole tubers could be the smooth cut after PEF treatment resulting in fewer fine particles and starch tending to burn on the surface of the potato slice (Ostermeier et al., 2021). According to a patent from Kalum and Hendriksen (2007), PEF treatment can also increase the efficacy of the asparaginase treatment on the raw product. Due to the potato cell membrane being perforated by PEF, the diffusion of the enzyme into the cell is increased, which also contributes to better contact with the substrate. Depending on the variety of parameters and technical characteristics of the company, PEF treatment can be easily applied in industrial potato processing with several benefits such as tissue softening, improvement of the cutting processes, reduction of oil uptake and thus economic savings (Fauster et al., 2018; Ostermeier et al., 2021).

Similarly to PEF, numerous studies have demonstrated the ability of US of high intensity (> 1 W/cm^2) and low frequency (20 to 150 kHz) can improve mass transfer through the formation of pores in cell envelopes and intense currents in the liquid environment, thereby favouring the diffusion of components (Chemat et al., 2011; Dourado et al., 2019). The general effect of US is to accelerate the softening process by hydrating the pectineus

material of the middle lamella, leading to the rupture of the plant tissue due to vibration (Toma et al., 2001). In addition, in plant-type cells, there are volatile compounds that could produce cavitation bubbles which also contribute to the rupture of the walls from the inside improving the leaching of compounds (Vinatoru et al., 1997). Therefore, integrating the application of high-intensity US to conventional water soaking accelerated the glucose and fructose extraction during the water soaking of potato slices, probably due to the formation of channels in the tissue which allowed a higher diffusion of reducing sugars (Maillard precursors) facilitating their leaching out (Antunes-Rohling et al., 2018; Esclapez et al., 2011; Pedreschi et al., 2021). In this respect, the application of US (480 W, 40 kHz) during water blanching of potatoes slices (60, 70 and 80 ◦C for 1, 8 and 15 min) significantly decreased the formation of AA in the final chips (Pedreschi et al., 2021). In detail, with US at 70 °C for 15 min, the greatest reduction of AA equivalent to 95% compared with the sample without any pre-treatment was reached thanks to a significant increase of sugars extraction rate (glucose: 60%, fructose: 30%). These results are coincident with those of Antunes-Rohling et al. (2018) who applied high-intensity US (35 and 130 kHz and, 9.5, 47.6 and 95.2 W/Kg, for 30 min at 30 and 42 °C) in a water bath as a frying pre-treatment for 30 min to reduce AA content in fried potato sticks avoiding undesirable texture and chemical changes in the final product. Also, in this case, the authors noted an increase in the extraction of reducing sugars, probably directed to the surface cells degradation and the chemical breakdown of starch. Recently, Ostermeier et al. (2021) investigated a promising combination of PEF pre-treatment and US-assisted frying, achieving a 66% reduction in AA level in potato chips. Due to the higher diffusion and water removal rate induced by PEF and US, the desired final moisture content was achieved more quickly without loss of product quality. By combining both treatments, a high evaporation rate is achieved, which reduces the frying time and can help to avoid temperature peaks on the product surface due to the more constant evaporation of water. Therefore, the low moisture content at the end of the frying process would be reached faster and the last frying period can be interrupted to allow less AA development (Ostermeier et al., 2021).

Another potential non-thermal technology that can be used as a treatment to produce fried potatoes with different/improved textural and nutritional properties, as well as for reducing the energy costs of some industrial steps of fried potato production is HHP (Aganovic et al., 2021; Dourado et al., 2020). Furthermore, as a pre-treatment combined with asparaginase immersion, HHP can be used as an AA mitigation strategy in fried

potato sticks due to the improved rate of enzyme infusion probably due to the structural changes caused in potato tissues and increased cell permeability, making the enzymatic process energetically and economically less expensive. Beyond that, HPP is described as a process that changes enzyme functionality, inactivating enzymes at higher pressures (> 400 MPa) and, in several cases, activating enzymes at lower pressures (< 200 MPa) (Eisenmenger & Reyes-De-Corcuera, 2009). Thus, it is possible that HHP could also induce the activation of asparaginase, but no information in the literature was found with regard to this enzyme. For the first time, Dourado et al. (2020) investigated this combination of treatments using HHP (100, 200 and 400 MPa for 5 min) and immersing raw potato sticks in asparaginase solution (10000 ASNU/L) with total enzymatic reaction times of 5, 10 and 20 min at room temperature. The potatoes pre-pressurized exhibited lower AA levels (181.1-246.8 µg/kg) than the respective control samples, independent of the enzymatic time and pressure level used in the pre-treatment. However, no significant AA changes were detected among samples treated at only 0.1 MPa, either immersed in asparaginase or water. Moreover, samples treated only by HHP, immersed in water without asparaginase (0 min of reaction time), exhibited AA levels in the range of 300.5 and 387.8 µg/kg. It means that HPP treatment alone (without asparaginase) was not enough to significantly affect the precursors of AA in raw potatoes and consequently was not effective to decrease significantly AA levels in fried potatoes (Dourado et al., 2020). Contrary to this finding, a previous study of Kobayashi et al. (2019), in an equimolar asparagine-glucose model system characterised by different pH and treated at 120 °C for 60 min within a range of 100 to 300 MPa, revealed suppression of AA generation. Probably, the high pressures suppressed the generation of AA, not initiating the reaction for other compounds but delaying part of the initial stage of the Maillard reaction. Based on these findings, the authors proposed the application of HHP to the heat processing of foods could be effective to control AA content.

The application of unconventional process techniques to remove AA after its formation, such as supercritical fluids technology (SFT) and cold plasma, could be also a strategy to control this undesired toxic compound (Nematollahi et al., 2021; **Paper I**). SFT is a gentle method that preserves the structural, nutritional and functional properties of food products (Casal et al., 2006). It is widely used to extract compounds in the food industry and has been successfully applied to coffee for the control of AA (Banchero et al., 2013). The efficiency of supercritical carbon dioxide (CO_2) extraction on AA removal ranged from 8 to 45% when the extraction time was below 525 min and reached a maximum of 79%

after 1305 min of supercritical fluid extraction treatment. The caffeine content of coffee beans was not affected by the treatment, but the effect on the organoleptic properties of the final product has not been evaluated, making further studies necessary. However, so far, no further investigations have been made, probably because the selection of the raw material and the preference for a darker roast are easier strategies to reduce AA in coffee. Recently, the use of cold plasma for toxin degradation in foods has also been proposed (Lee et al., 2020). Cold plasma is a novel technology, which uses reactive compounds in plasma to damage cell membranes, denature the DNA and, consequently, destroy or inactivate microorganisms. As cold plasma is a cold treatment, the sensory and functional properties of foods have the potential to be better maintained (Dey et al., 2016). Lee et al. (2020) treated the roasted coffee with corona discharge plasma jet (CDPJ) for 15, 30, 45 and 60 min generated under atmospheric pressure conditions using a 1.5 A current (input) at 58 kHz operating frequency. The concentrations of AA were decreased in the beans in a time-dependent manner, initial concentrations (35.4 μg/kg) were reduced by about 30% after 60 min. In addition, the authors reported that sensory characteristics and caffeine concentration of roasted coffee beans were not negatively affected by CDPJ treatment. Although cold plasma has several benefits, some disadvantages have been also to be considered and investigated, namely, the negative effect regarding the lipid oxidation adversely affects the sensory and nutritional quality of some foods products (Gavahian et al., 2018).

Potential innovative methods to reduce AA levels are performed by eliminating its precursors, intervening with the Maillard reaction, or removing AA after its formation. A combination of conventional methods and emerging techniques is also an efficient AA mitigation strategy. However, few studies on these new procedures have been conducted so far, particularly on supercritical fluid and cold plasma technologies; however, other techniques like PEF are already widely utilized and tested in the food sector.

Chapter 3

Research outcomes: mitigation of acrylamide by application of pre-treatments

This chapter was based on **Paper II**:

Schouten M.A.; Genovese J.; Tappi S.; Di Francesco A.; Baraldi E.; Cortese M.; Caprioli G.; Angeloni S.; Vittori S.; Rocculi P.; Romani S. (2020). Effect of innovative pre-treatments on the mitigation of acrylamide formation in potato chips. *Innovative Food Science and Emerging Technologies*, 64, August 2020, 102397, pp. 1-11 (Available online 29 May 2020, doi.org/10.1016/j.ifset.2020.102397).

3.1 Effect of pulsed electric fields and yeast dipping in potato chips

3.1.1 Introduction and aim of the research activity

Potatoes are the second and fourth most important crops for human consumption in Europe and the world, respectively, and are a good source of energy, carbs and nutritional fibres (Dourado et al., 2019). Nevertheless, because of the asparagine and reducing sugars content, as well as the high temperatures used during the frying process and the high surface to volume ratio, potato snacks (i.e. French fries, crips, etc.) are especially vulnerable to AA formation (Parker et al., 2012). For the potato products classified as "potato chips made from fresh potatoes and potato dough", according to Commission Regulation (EU) 2017/2158, food business operators, should apply mitigation measures to ensure a minimum formation of AA below the reference level set at 750 µg/kg (European Commission, 2017).

The main conventional strategies proposed to reduce AA in potato products are the selection of cultivar and storage conditions, the control of time and temperature of heat treatment, the application of alternative frying techniques (e.g. under vacuum), the use of asparaginase enzyme and hot water blanching as pre-treatments (Amrein et al., 2003; Foot et al., 2007; Pedreschi et al., 2011; Romani et al., 2009). However, these strategies present different drawbacks including long processing times, high costs, negative sensory alterations as well as difficult implementation for industrial scale making it necessary to find alternative methods to reduce the generation of AA in potato products.

The application of biocontrol agents as asparaginase producers and non-thermal treatments such as pulsed electric fields (PEF) were proven useful to reduce the AA precursors in the potato tissues and hence the subsequent AA development (Di Francesco et al., 2019; Genovese et al., 2019). Di Francesco et al. (2019) reported that the yeast *Aureobasidium pullulans* L1 strain can successfully assimilate asparagine in potato homogenate after 30 min of contact, leading to a great decrease in the AA content in the final fried potato crips (−85%). The metabolic activity of microorganisms could reduce the asparagine concentration through the activity of the asparaginase enzyme. The yeast ability to produce enzymes has attracted considerable biotechnological interest because these hydrolytic enzymes have a potential commercial value in various industries (Deshpande, Rale and Lynch, 1992). Moreover, the yeast *A. pullulans* has been demonstrated to be able to ferment sugars, among which sucrose covers an important role because proved as the carbon source for pullulan synthesis (Sheng et al.,

2016). Furthermore, An et al. (2017) showed how potato starch can promote *A. pullulans* enzyme production and pullulan biosynthesis. Regarding PEF treatment, Genovese et al. (2019) described the possibility to reduce AA precursors in raw potatoes by the application of PEF followed by a 5 min water dipping, which led to a reduction of around 30% of AA content in fried potato crips.

The current research activity aimed to evaluate the possibility to reduce AA in potato crips by applying for different times the innovative pre-treatments, *A. pullulans* L1 strain and PEF, alone or in combination. The AA and its precursors (e.g. reducing sugar and asparagine) content, quality parameters such as moisture, oil content, colour and texture of potato crips were also evaluated.

3.1.2 Materials and methods

3.1.2.1 Potato sample preparation and pre-treatments

Fresh potato tubers (*Solanum tuberosum* cv Lady Claire) were purchased at the local market (Cesena, Italy) and stored in the dark at 15 °C and at 90% relative humidity. Tubers were washed in running water, manually peeled and cut in slices of 1.5 ± 0.2 mm thickness by using an electric cutter machine mod. KAFPL0922N (CAD Italy, Mareno di Piave, Italy). Potato slices were rinsed immediately after slicing in tap water (18 ± 2 °C) to eliminate the starch material on the surface.

Before frying, the raw potato slices were subjected to different pre-treatments summarised in **Figure 3.1**. In detail, the pre-treatments were: dipping in water for 5 and 15 min (samples named respectively W5 and W15); dipping in *A. pullulans* L1 strain yeast water suspension (10^8 cells/mL) for 5 and 15 min (Y5 and Y15 samples); dipping in water after PEF in water (PW5 and PW15 samples) and dipping in yeast water suspension after PEF in the same yeast suspension (PY5 and PY15 samples). Raw potato slices that have not undergone any pre-treatment were considered as a control sample (C).

The yeast pre-treatments were carried out according to the patented procedure No. WO2019058248A1 (authors: M. Mari, A. Di Francesco and L. Ugolini, *Alma Mater Studiorum* – University of Bologna and CREA), with slight modifications. Each treatment was carried out at room temperature (about 25 °C) by dipping 80 g of potato slices in 200 mL of yeast suspension concentrated 10^8 cells/mL for 5 (Y5) and 15 (Y15) min. The yeast untreated samples were represented by 80 g of potato slices dipped in 200 mL of tap water at room temperature for the same times (5 min: W5, 15 min: W15). After dipping,

the potato slices were collected, rinsed with tap water and carefully dried with absorbent paper. Each treatment for each sample (W5, W15, Y5, Y15) was performed in triplicate. The PEF pre-treatments were performed using a lab-scale PEF unit mod. SeP7500 equipped with a prototype treatment chamber (Alintel, Pieve di Cento, Italy).

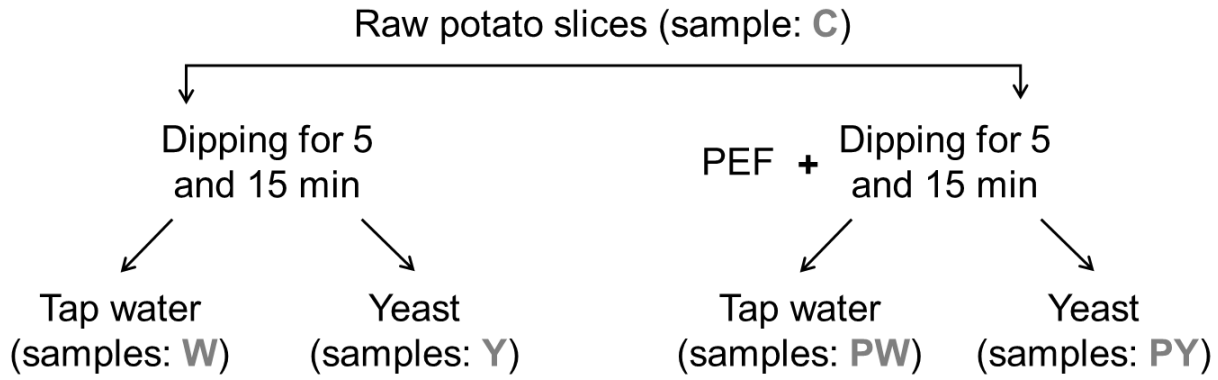


Figure 3.1 - Representation of the studied pre-treatments for the acrylamide reduction in fried potato chips and corresponding samples codes.

Table 3.1 shows the settings and parameters used for the application of electric fields. The outputs of the generator, tension and current, were monitored using a PC-oscilloscope mod. Picoscope 2204a (Pico Technology, St Neots, UK). Raw potato slices (20 g) were treated at room temperature (about 25 °C) in tap water (100 mL) with an initial electrical conductivity of 536 ± 23 µS/cm (mod. Basic 30, Crison, Barcelona, Spain). For more details regarding the PEF-treatment protocol and the measured cell disintegration, refer to the protocol of Genovese et al. (2019). The PEF treatment was repeated four times to obtain one batch (80 g) of treated product for each sample. After the treatments, 80 g of potato slices were collected and dipped in 200 mL of water for 5 (PW5) and 15 (PW15) min. After dipping, the potato slices were collected, rinsed with tap water and carefully dried with absorbent paper. Each dipping treatment was performed in triplicate for each sample (PW5 and PW15), consequently, the preliminary PEF treatment was repeated 12 times for each sample ((20 g × 4) ×3).

The combination of PEF and yeast pre-treatments was performed filling the PEF treatment chamber with 100 mL of yeast aqueous suspension (10^8 cells/mL) and 20 g of raw potato slices. The initial electrical conductivity of the yeast aqueous suspension was measured using an electrical conductivity meter mod. Basic 30 (Crison, Barcelona, Spain), was 536 ± 23 µS/cm at 25 °C (comparable with the tap water initial electrical conductivity used for PEF treatment in water). The selected PEF conditions and applied

energy input were the same as previously mentioned. After the PEF treatments, 80 g of potato slices were collected and left immersed in 200 mL of the PEF-treated yeast suspension for 5 (PY5) and 15 (PY15) min. Subsequently, the potato slices were collected, rinsed with tap water and carefully dried with absorbent paper. Each dipping treatment in the PEF-treated yeast suspension was performed in triplicate for each sample (PY5 and PY15); consequently, the preliminary PEF treatment in the yeast aqueous suspension was repeated 12 times for each sample ((20 g × 4) × 3).

Table 3.1 - Settings and operating parameters used in the PEF pre-treatment of raw potato slices.

Parameters	Value
Electric field strength (kV/cm)	1.5 ± 0.2
Number of pulses	1000
Distance between electrodes d (cm)	4.7
Pulse-type	Rectangular monopolar
Fixed pulse width τ (μs)	10
Time interval between pulses (ms)	10 ± 1
Frequency (Hz)	100
Treatment time (s)	10
Energy input (kJ/kg)*	105 ± 5.5

* calculated according to Raso et al. (2016).

Using an electrical fryer mod. MFR280R (Fama Industrie, Rimini, Italy), all untreated and pre-treated potato slices were deep-fried in 6 L of high-oleic sunflower oil with a potato to oil ratio of 1:20 w/w at 175 °C for 3 min. Temperatures in the frying oil were measured using K-type thermocouples (mod. Chromel/Alumel, Tersid, Sesto San Giovanni, Italy) coupled to a data recording system mod. 9211A (National Instruments, Austin, TX, USA).

3.1.2.2 Isothermal calorimetry pre-trials

Isothermal calorimetry was used to evaluate the best combination of PEF and dipping in aqueous yeast suspension pre-treatments, by monitoring the development of metabolic heat of the *A. pullulans* L1 yeast. In detail, Nutrient Yeast Dextrose Broth (NYDB) medium was used as the ideal substrate for yeast growth, while tap water and potato tissue was used to simulate a real substrate. Moreover, because PEF could also increase the release of solutes from the potato tissue that could influence the yeast activity, tap water with potato tissue subjected to PEF was also considered. In each vial, 2 g of potato (raw or

PEF-treated), 1 mL of yeast (10^8 cells/mL) (control and PEF-treated) and 2 mL of substrate (NYDB or tap water) were placed. The vials were sealed with Teflon caps and aluminium screw lids. The extent of metabolic heat production by the yeast was measured continuously with a TAM Air isothermal calorimeter (TA Instruments/Thermometric, Järfälla, Sweden) with a sensitivity of ± 10 μW and recorded with dedicated software (TAM Air assistant, TA Instruments/Thermometric, Järfälla, Sweden). As a reference, a vial with distilled water was used according to Panarese et al. (2012). Isothermal calorimetric measurements, for 48 h, were performed at a constant temperature of 25 °C, as the optimal one for yeast growth. The thermograms obtained were normalized for the weight of the sample and three replicas were performed for each sample.

3.1.2.3 Asparagine, reducing sugars and acrylamide quantification

The sample extraction process was optimized by taking the cue from a previous study with some modifications (Nielsen et al., 2006). Water was used as the extraction solvent for the high solubility of target molecules and a dispersive solid-phase extraction (DSPE) using C18 sorbent was chosen for sample clean-up because was a simple and fast technique able to remove non-polar molecules which could act as interferences (Anastassiades et al., 2003). In detail, the freeze-dried raw potatoes and potato chips samples were crumbled finely in a mortar and 2 g of sample were weighed in a 50 mL conical flask. The extraction was performed with 20 mL of Milli-Q water firstly by 1 min of agitation in a vortex mixer and secondly by 10 min of ultrasound-assisted extraction at room temperature. After pouring into a 50 mL centrifuge plastic tube, the sample was centrifuged at 5000 rpm for 10 min and the supernatant was collected and stored overnight at −18 °C to precipitate starch and facilitate the removal of the fat fraction. Later, the sample was thawed at room temperature (about 25 °C), once again centrifuged at 5000 rpm for 10 min and then 1 mL of water supernatant was transferred to a 1.5 mL microcentrifuge tube containing 100 mg of C18 sorbent. Before centrifugation at 13300 rpm for 15 min, the sample was vortexed for 1 min. Finally, an aliquot of the supernatant was collected and diluted 1:100 in the mobile phase for asparagine analysis and 1:2 in acetonitrile and filtered with a 0.2 μm polytetrafluoroethylene (PTFE) syringe filters for AA, fructose and glucose analysis.

HPLC-MS/MS analysis was performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an electrospray ionisation (ESI) source operating in positive ionisation mode, meeting the

limits of detection (LOD) and quantification (LOQ) required by European Commission Regulation (EU) 2017/2158. The HPLC-MS/MS parameters of each analyte were optimized in flow injection analysis (FIA) (1 µL of a 10 mg/L individual standard solution) by using optimizer software (Agilent, Santa Clara, CA, USA). The separation of target compounds was achieved on a Kinetex Hilic analytical column (2.6 µm, 100 mm × 4.6 mm) from Phenomenex (Torrance, CA, USA) preceded by a KrudKatcher ULTRA HPLC In-Line Filter (2.0 µm Depth Filter × 0.004 in ID). The mobile phase for HPLC-MS/MS analysis was a mixture of 15% water (A) and 85% HPLC-grade acetonitrile (B), both with 0.1% formic acid. The separation was obtained by flowing at 0.8 mL/min with the following gradient elution: isocratic condition until 2.5 min (85% B), 3.5 min (70% B), 5.5 min (70% B), 6.5 min (85% B) and then constant until the end of the run (15 min). All solvents and solutions were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 µL. The temperature of the column was 25 °C and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 12 L/min, the nebulizer pressure was 45 psi and the capillary voltage was 4000 V. Detection was performed in the multiple reaction monitoring (MRM) mode. The MRM peak areas were integrated for quantification and the most abundant product ion was used for quantitation, while the rest of the product ions were used for qualification. The selected ion transitions and the mass spectrometer parameters are reported in **Table 3.2**. All chemicals and reagents were of analytical grade. Results were expressed as µg/kg for AA and mg/kg for AA precursors on dry matter basis. Three measurements were taken for each sample type.

Table 3.2 - HPLC-MS/MS acquisition parameters of the MRM mode adopted for the quantification of acrylamide and its precursors.

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Fragmentor (V)	Collision energy (V)
Asparagine	133.00	98.00	5.17	64	16
Fructose	203.00	203.00	1.96	80	0
Glucose	203.00	203.00	2.18	80	0
Acrylamide	72.00	55.00	1.52	45	8

3.1.2.4 Main quality parameters analysis

For each potato sample, the following analytical determinations were carried out:

- moisture (%) of raw and fried potatoes, determined on 3 g exactly weighed of ground sample by drying up to constant weight in a thermoregulated laboratory oven mod. UF110 (Memmert, Schwabach, Germany) at 70 °C and 105 °C, respectively;
- oil content (%), determined on the dried potato chips by Soxhlet extraction, performing the procedure with petroleum ether as solvent at 60 °C for 3 h (AOAC, 2000);
- colour of potato chip surfaces, measured using a computer vision system (CVS) consisting of a dark chamber with a white background, four daylight fluorescent lamps (TL-D Deluxe, Natural Daylight, 18 W/965, Philips, Amsterdam, The Netherlands) with a colour temperature of 6500 K (D65 standard). The RGB images of the samples were acquired using a colour digital camera mod. D7000 (Nikon, Tokyo, Japan) equipped with a 105 mm lens (mod. AF-S Micro Nikkor, Nikon, Tokyo, Japan), positioned vertically and connected to display and capture the images directly by the computer. The pre-processing of RGB images and colour quantification in the CIE L*a*b* scale was performed with ImageJ analysis software (NIH, Rockville Pike, MD, USA). From numerical values of a* (green-red) and b* (yellow-blue) chromatic parameters, hue angle was calculated ($h° = (\tan^{-1}(b^*/a^*)/2\pi)\cdot 360$) (McGuire, 1992);
- texture of potato chips, measured at room temperature using a Texture Analyser mod. TA-HDi500 (Stable Micro System, Surrey, UK) equipped with a 5 kg load cell and a spherical probe in stainless steel with a diameter of 6 mm at 1.0 mm/s test speed. The samples, selected based on uniform size and shape, were placed on a support rig (HDP/CFS) and compressed for a 3 mm distance. The acquired results were expressed as hardness, calculated through maximum force (N) values, and an index of crispness, calculated by means of the linear distance between the first and the last peaks registered (Tylewicz et al., 2019).

All analyses were performed in triplicate for each sample, except for the determination of colour and texture, which were evaluated on both surfaces (upper and lower) of 5 chips per sample and 12 chips per sample, respectively.

3.1.2.5 Data analysis

The results were reported as mean value ± standard deviation of replications. The data processing and statistical analysis were performed in Excel (Microsoft, Redmond, WA, USA) and STATISTICA 8.0 software (StatSoft Inc., Tulsa, UK). Significant differences between results were calculated by unidirectional analysis of variance (ANOVA) and Tukey's post-hoc comparison test, with a significance level of 95% ($p < 0.05$).

3.1.3 Results and discussion

3.1.3.1 Optimization of combined pre-treatments

Isothermal calorimetry, which has been successfully used to describe the growth ability of microorganisms (Braissant et al., 2010), was successfully used to measure the heat flow produced in different substrates by the yeast *A. pullulans* L1, untreated and subjected to the PEF treatment. At first, the ideal growth substrate (NYDB) was used to evaluate the only effect of the selected PEF conditions on the yeast activity in terms of heat produced. **Figure 3.2A** shows the thermal power, recorded at 25 °C during 35 h, produced by *A. pullulans* after PEF treatment (YPEF) compared to the untreated one (Y). The thermograms, as suggested by Morozova et al. (2017), are relative to the kinetic profile of the fermentative process and index of the yeast growth rate.

The shape of the signal suggests the presence of a series of consecutive processes that occur in the vial. Initially, in both samples, a constant heat flow was recorded, of about $8.4 \cdot 10^{-4}$ and $2.0 \cdot 10^{-3}$ W/g for sample Y and YPEF respectively, indicating the yeast lag phase. Typically, the lag phase corresponds to the period of time in which yeasts synthesize the enzymatic pool necessary for their catabolism during which the multiplication is neglectable (Morozova et al., 2017). After about 10 h, for both samples, the heat signal started to increase exponentially due to the exponential growth phase of the yeast. However, the signal increased until reaching a maximum, in different times for the two samples, and then, because of various factors limiting the growth (e.g. reduced oxygen concentration in the headspace, reduction of carbon and nitrogen sources in the growth medium, production of ethanol or increased pressure in the vial), the signal started decreasing, reaching almost a stationary phase, the extension of which depends on the ability of the yeast to survive (Morozova et al., 2017).

The average values of the heat produced by the yeast according to the different conditions investigated, expressed as either total heat (integral of the thermal power) and slope of the curve related to the exponential growth phase, are reported in **Table 3.3**. Results obtained suggest that PEF treatment had a positive effect on the yeast metabolism stimulating both its entity and rate. Different literature studies indicated that low-intensity electrical fields (i.e. 0.1-1 kV/cm) and short treatment times (μs) did not bring damage to the cell membranes functionality, but enhanced microbial reactions and activities (Mattar et al., 2014; Schottroff et al., 2017).

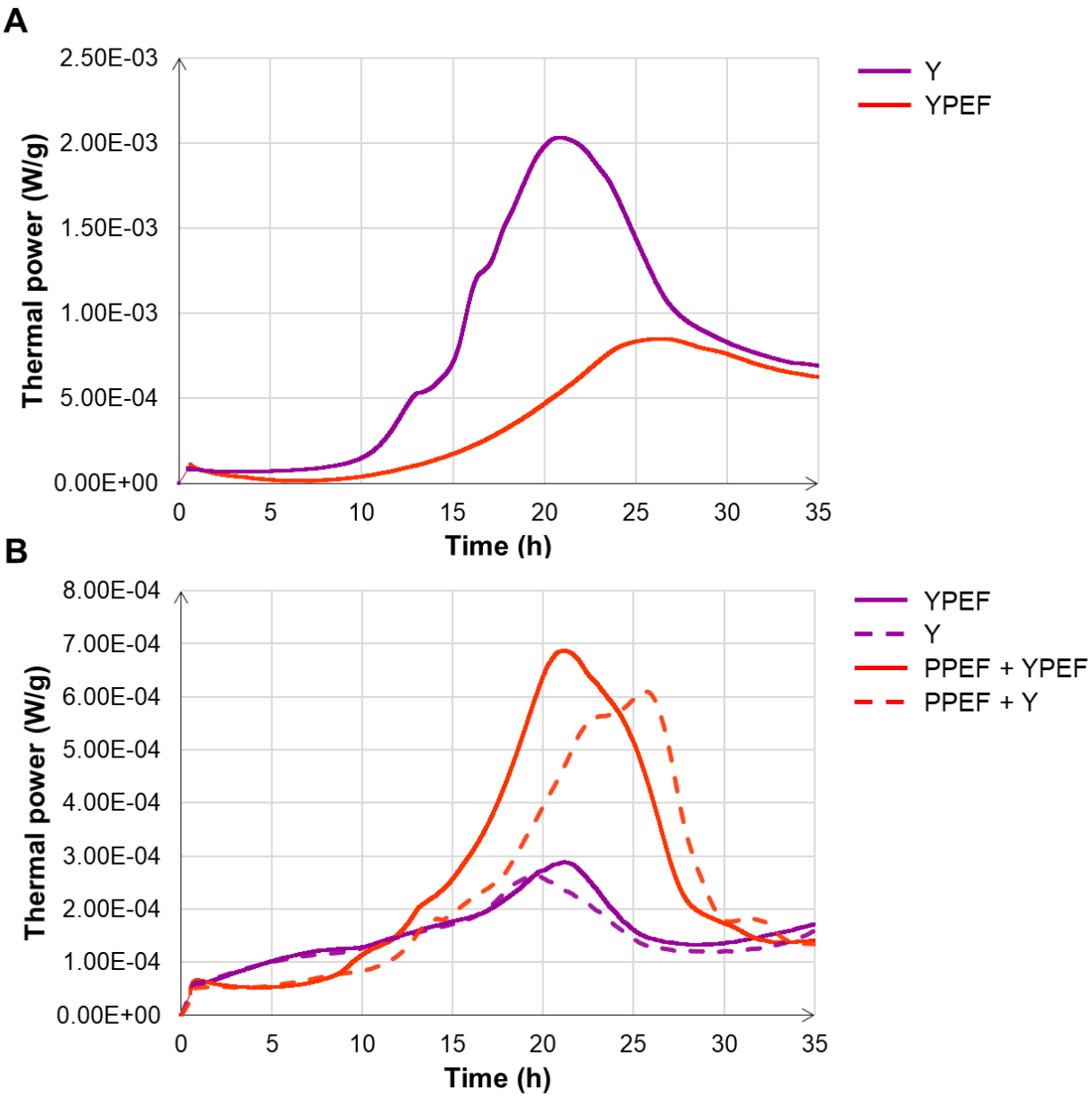


Figure 3.2 - Thermal profiles of the *A. pullulans* L1 yeast during PEF treatment, compared to control growth in an ideal (A) and real (B) substrate, as assessed by isothermal calorimetry (Y: untreated yeast; YPEF: PEF-treated yeast; PPEF: PEF-treated potato).

According to these investigations, low-intensity PEF allows to improve yeasts enzymatic synthesis, frequency of cell division, probability of survival of daughter-cells, increases tolerance to inhibitor conditions and fermentation ability. In addition, electroporation induced by PEF can modify the cytoplasmic membrane and hence the transportation of the nutrients due to the formation of pores or the activation of transport proteins. However, such mechanisms have not been fully clarified yet and PEF effect depends also on other factors such as growth substrate, size and specific resistance of microbial cells (Mattar et al., 2014). In relation to the different susceptibility of different microorganisms and the

entity of PEF treatment applied, the cells can be in three different states: intact, dead or damaged (sub-lethal stress) (M. Wang et al., 2018).

Table 3.3 - Calorimetric heat (J/g) and slope (W/g·h) measured during the *A. pullulans* L1 yeast growth in ideal and real substrates after PEF treatment compared to the control (Y: untreated yeast; YPEF: PEF-treated yeast; PPEF: PEF-treated potato).

Sample	Heat (J/g)	Slope (W/g·h)
Ideal substrate: nutrient yeast dextrose broth		
Y	24.7 ± 2.8^{a}	$5.5 \pm 0.7 \cdot 10^{-5a}$
YPEF	45.8 ± 0.7^{b}	$2 \cdot 10^{-4} \pm 0.1^{b}$
Real substrate: potato + water		
Y	17.9 ± 0.7^{A}	$1 \cdot 10^{-5} \pm 0.1^{A}$
YPEF	19.0 ± 0.9^{A}	$1.5 \pm 7 \cdot 10^{-5A}$
PPEF + Y	31.6 ± 4.0^{B}	$6.5 \pm 4 \cdot 10^{-5B}$
PPEF + YPEF	33.2 ± 2.3^{B}	$5.0 \pm 1 \cdot 10^{-5B}$

Different letters (lowercase or uppercase) in the same column indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Figure 3.2B shows the thermal power signals recorded at 25 °C during the growth of the yeast subjected to PEF (YPEF) compared to the control (Y), placed in a substrate simulating the real conditions, consisting of water and untreated potatoes or water and potatoes treated with PEF (PPEF + Y and PPEF + YPEF). The corresponding data of heat produced and the slope of the curves were reported in **Table 3.3**. As expected, the extent of yeast growth in the water-potato medium was significantly reduced compared to its optimal growth medium (NYDB). However, when placed in a water-potato medium, no difference was observed for the yeast treated by PEF (PPEF + YPEF) compared to its untreated control (PPEF+ Y) for both heat and slope produced. Nevertheless, when the potato in the substrate was pre-treated by PEF (PPEF+Y and PPEF+YPEF), the heat produced almost doubled and the slope increased 5/6-fold in comparison to water-potato medium not treated with PEF (Y and YPEF). While total heat is an index of the metabolism entity, the slope indicates the rate of metabolic reactions. The hypothesis is that PEF pre-treatment improved the membrane permeability of the potato tissue, resulting in a larger release of the cell content into the water, which became richer in nutrients accessible by yeast metabolism. Moreover, in the water-potato medium (real substrate) no differences in heat and slope were observed between PEF-treated (YPEF) and control (Y) yeast in

both potato mediums treated with PEF or not (**Table 3.3**). It seems that the positive effect of PEF on *A. pullulans* metabolism is visible only when the growth substrate is NYDB (**Figure 3.2A**), while in the case of the water-potato substrate, the limiting nutrient availability flattened the differences (**Figure 3.2B**).

Following these preliminary results, it was decided to PEF-treat the potato slices in the yeast water suspension to verify the combined effect of the two pre-treatments on AA formation.

3.1.3.2 Influence of pre-treatments on acrylamide and precursors contents

The levels of AA in fried potato chips and the concentrations of AA precursors in untreated control raw potato (C) and differently pre-treated raw potato samples are indicated in **Table 3.4**.

Table 3.4 - Acrylamide (µg/kg) level expressed on dry matter (d.m.) basis of fried potato chips and AA precursors (mg/kg) content on d.m. basis of raw potato samples (C: control; W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

Sample	Time (min)	Acrylamide (µg/kg d.m.)	Asparagine (mg/kg d.m.)	Glucose (mg/kg d.m.)	Fructose (mg/kg d.m.)
C		1384.3 ± 65.0^{a}	8826.3 ± 576.0^{b}	33.6 ± 6.8^{abc}	8.4 ± 1.0^{c}
W	5	1292.4 ± 96.2^{bc}	12952.0 ± 1341.9^{a}	19.6 ± 0.4^{d}	6.9 ± 0.7^{d}
	15	775.3 ± 81.5^{d}	12036.4 ± 2142.5^{a}	29.50 ± 5.0^{c}	7.8 ± 1.1^{cd}
Y	5	1375.9 ± 9.9^{ab}	12945.3 ± 441.7^{a}	39.5 ± 4.1^{a}	16.4 ± 0.7^{a}
	15	676.4 ± 42.3^{e}	5957.5 ± 135.6^{c}	18.6 ± 0.9^{d}	3.8 ± 0.2^{f}
PW	5	886.8 ± 9.9^{d}	4108.9 ± 571.3^{c}	36.7 ± 0.88^{ab}	5.0 ± 0.1^{ef}
	15	572.0 ± 8.8^{f}	5875.0 ± 695.9^{c}	17.8 ± 3.0^{d}	5.4 ± 0.4^{e}
PY	5	1211.9 ± 4.2^{c}	4188.0 ± 69.9^{c}	33.53 ± 1.2^{abc}	5.7 ± 0.2^{e}
	15	1193.2 ± 20.1^{c}	4360.8 ± 87.9^{c}	31.1 ± 1.2^{bc}	14.1 ± 0.5^{b}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The AA concentration of untreated potato chips was 1384.3 µg/kg, a value that exceeds the reference level set by the last Commission Regulation (EU) 2017/ 2158 (750 µg/kg). The untreated potato samples were characterized by asparagine, glucose and fructose contents of 8826.3 mg/kg, 33.6 mg/kg and 6.8 mg/kg, respectively.

Each applied pre-treatment, especially after 15 min of dipping, has led to a reduction of AA in final potato chips; however, not always proportional to the concentration of evaluated AA precursors. Dipping in water (W5 and W15) had a positive effect in reducing AA levels in fried potatoes due to the promotion of precursors release from the tuber matrix to the water for leaching effect. Potato samples dipped in water for 5 and 15 min (W5 and W15) have presented a significant AA reduction respectively of 6.6% and 44.0%. The decrement of AA in these samples can be attributed to the reduction of glucose and fructose, while the asparagine content did not undergo any reduction. It is worth to note that the sample W15 resulted in AA content in compliance with the limit set by the EU Regulation (750 µg/kg). Nevertheless, the concentration of AA precursors, and so the potential concentration of AA formation, is linked to intrinsic properties of the raw material, e.g. potato cultivar and post-harvesting conditions. Therefore, AA levels in fried potatoes can be much higher than those measured in this study, explaining the necessity of assessing new AA mitigation measures appropriate for industrial processing conditions.

The yeast treatment after 5 min (Y5) did not lead to a significant reduction in AA compared to the control (C) and the water-dipped sample for the same time (W5). On the other hand, after 15 min (Y15) a significant AA reduction of 51.1% was reached, highlighting that the yeast requires contact times more than 5 min. Nevertheless, in comparison to the sample dipped in water for the same time (W15) the additional reduction after 15 min yeast treatment (Y15) was only 7%. This slight, but significant reduction of AA after 15 min yeast dipping could be attributed to the enzymatic and metabolic activity of the yeast able to reduce the levels of glucose, fructose and asparagine. In a study conducted with the same yeast strain, a reduction of AA in potato chips, equal to 83.7%, was obtained after dipping of 30 min (Di Francesco et al., 2019). However, an industrial application often requires shorter times. The samples subjected to PEF treatment followed by dipping in water (PW) led to the highest AA reduction for each treatment time, equal to 35.9% and 58.7% respectively for 5 and 15 min. The cell electroporation phenomena induced by the PEF treatment on raw potato slices resulted in a reduction of AA precursors leading to lower AA formation. This result agrees with Genovese et al. (2019) who found a significant reduction in AA content (30%) in PEF pre-treated chips compared to untreated samples.

Moreover, it is interesting to notice that, although precursors' concentration was similar for Y15 and PW15 samples, AA levels were lower in PW15. This could be attributed to the possible modification of other molecules and pathways which can participate in AA

formation. In fact, it is known that AA is mainly formed by Maillard reaction from asparagine and α-carbonyl sources such as reducing sugars, but other mechanisms can contribute to the final AA level. For example, acrolein, which can be formed from lipid oxidation and degradation of amino acids, carbohydrates and proteins, the acrylic acid derived from aspartic acid and the β-alanine can participate in AA formation (Stadler & Studer, 2015).

The combination of PEF and yeast treatments (PY) resulted in a slight AA reduction (12.4% after 5 min and 13.8% after 15 min), similar to the results obtained with 5 min of dipping in water, but significantly lower compared to the reductions obtained with the singular innovative pre-treatments (Y15, PW5 and PW15). This result could be related to the direct effect of PEF on yeast cells that can notably influence their propriety and activity. In fact, as reported by Stirke et al. (2014) and Mattar et al. (2015) PEF can influence yeast cell viability, size and, consequently, their enzymatic activity. Although isothermal calorimetry results showed an increase in the overall yeast metabolic activity, the specific alterations of metabolic pathways induced by PEF are still unknown (Mattar et al., 2015).

3.1.3.3 Influence of pre-treatments on the main qualitative parameters

The development of non-enzymatic browning reactions (i.e. Maillard reaction), that occur during deep fry heat treatments, are responsible for the formation of undesirable AA formation, as well as desired alterations in physical qualities such as colour, texture, flavour, moisture and oil content (Pedreschi et al., 2005; Romani et al., 2009; Y. Yang et al., 2016).

The main quality characteristics of fried potato samples were analysed to evaluate the possible effect of different AA mitigation pre-treatments applied. The colour parameters, texture values, moisture and oil content of samples are reported in **Tables 3.5** and **3.6**.

The development of a brown-gold colour during potato frying is one of the most important quality parameters demanded by consumers (Medeiros Vinci et al., 2012). The use of the CVS analysis allowed to evaluate the colour of the whole potato chip surfaces. Examples of RGB images of potato chips samples untreated and differently treated are shown in **Figure 3.3**, each RGB image was processed to extract the numerical CIE L*a*b* data. The W15, Y15, PW5, PW15 and PY15 potato chips presented significant ($p < 0.05$) higher L* values compared to the other samples, highlighting a brighter colour.

Table 3.5 - Colour and texture parameters of untreated (C: control) and treated potato chips (W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

Sample	Time (min)	Lightness (L*)	Hue angle (h°)	Hardness (N)	Crispness (linear distance)
C		84.5 ± 1.1^{b}	93.2 ± 1.7^{ef}	3.9 ± 1.1^{a}	9.3 ± 2.6^{a}
W	5	84.5 ± 1.2^{b}	94.6 ± 0.9^{de}	2.9 ± 0.4^{bc}	8.0 ± 1.7^{ab}
	15	87.1 ± 0.2^{a}	94.9 ± 0.6^{cd}	3.6 ± 0.8^{ab}	8.8 ± 2.6^{ab}
Y	5	84.3 ± 1.2^{b}	92.8 ± 0.8^{f}	3.7 ± 0.8^{ab}	9.7 ± 1.5^{a}
	15	86.0 ± 1.3^{ab}	96.4 ± 1.1^{bc}	3.3 ± 0.6^{abc}	9.7 ± 2.4^{a}
PW	5	86.1 ± 0.7^{ab}	98.9 ± 0.3^{a}	2.6 ± 0.7^{bc}	6.6 ± 2.1^{b}
	15	86.4 ± 1.1^{ab}	97.8 ± 0.9^{ab}	3.1 ± 0.9^{bc}	9.1 ± 2.2^{a}
PY	5	84.7 ± 1.3^{b}	93.5 ± 0.2^{def}	2.9 ± 0.7^{bc}	7.6 ± 2.9^{ab}
	15	85.2 ± 0.8^{ab}	93.4 ± 0.2^{def}	3.0 ± 0.8^{bc}	8.8 ± 2.8^{ab}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Table 3.6 - Moisture (%) and oil content (%) of raw* and fried potato chips (W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

Sample	Time (min)	Moisture* (%)	Moisture (%)	Oil (%)
C		74.1 ± 0.5^{b}	4.1 ± 0.1^{ab}	30.3 ± 1.3^{d}
W	5	78.9 ± 0.4^{a}	4.2 ± 0.1^{a}	37.3 ± 0.1^{b}
	15	79.9 ± 0.8^{a}	4.1 ± 0.1^{ab}	34.3 ± 0.6^{c}
Y	5	78.1 ± 0.7^{a}	4.0 ± 0.4^{ab}	34.7 ± 0.8^{c}
	15	79.2 ± 0.2^{a}	3.7 ± 0.1^{b}	33.5 ± 0.6^{c}
PW	5	74.5 ± 1.3^{b}	2.8 ± 0.1^{c}	41.3 ± 0.9^{a}
	15	75.6 ± 0.9^{b}	2.7 ± 0.3^{c}	37.5 ± 0.8^{b}
PY	5	74.5 ± 0.9^{b}	2.5 ± 0.2^{c}	41.9 ± 0.8^{a}
	15	75.0 ± 0.4^{b}	2.5 ± 0.4^{c}	38.4 ± 0.5^{b}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The h° values increased significantly for W15, Y15, PW5 and PW15 samples, underling a colour variation from the red-orange (dark) zone to the green-yellow (light) one compared to the untreated sample (C).

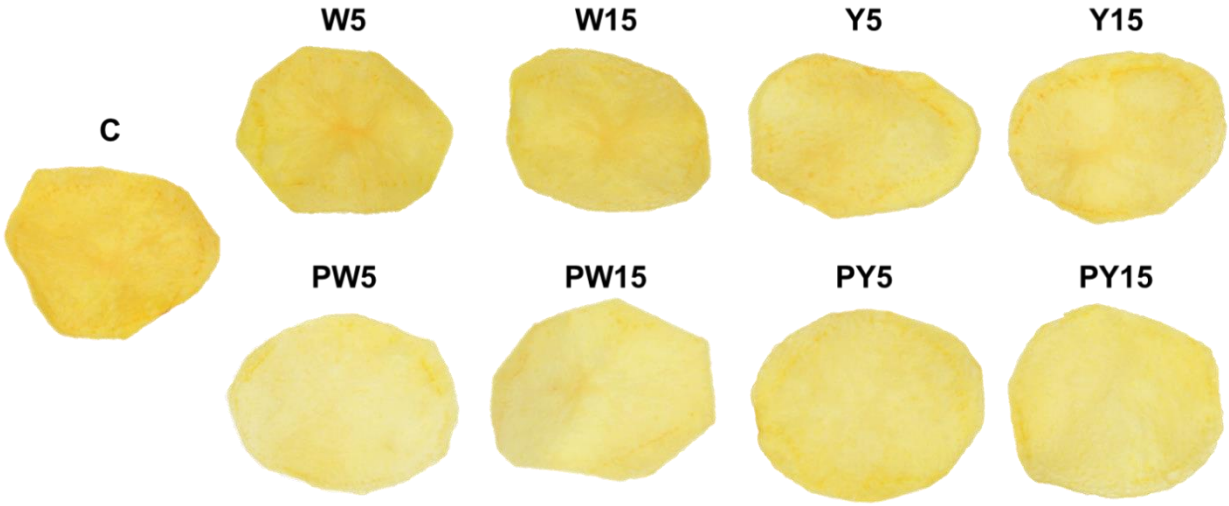


Figure 3.3 - Examples of RGB images of potato chips samples untreated (C: control) and differently treated (W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

The texture is another important sensorial characteristic of potato chips that should be monitored (Yee & Bussell, 2007). The various pre-treatments studied led to a slight and not always significant reduction of hardness and crispness index of potato chips compared with the untreated sample (C). Very few studies were carried out on the structural characteristics of the potatoes after similar pre-treatments. Zhou et al. (2015), using a different potato variety, found that the untreated potato samples were darker than yeast-treated ones characterized by a low AA content. However, no significant ($p>0.05$) differences in texture were found among the treated fried potato samples and untreated ones. The colour and texture results were also confirmed by the study of Di Francesco et al. (2019) who found that the potato untreated samples appeared darker in colour than yeast treated ones. Regarding the effect of PEF treatment, Ignat et al. (2015) observed significant differences only in L* and a* chromatic values for PEF-treated fried potato cubes, while the hardness was similar to the control sample. Genovese et al. (2019) discovered similar colour development when frying of PEF-treated potato slices, confirming these findings. Furthermore, the authors discovered that PEF-treated samples had a minor but substantial drop in textural characteristics when compared to untreated samples, as in this case.

In the raw potato samples, the water dipping treatments led to a significant moisture content increase for all treatment times (W5 and W15) compared to the untreated control sample (C) probably due to capillary and osmotic phenomena (**Table 3.6**). No significant ($p > 0.05$) differences were found between the moisture values of the samples subjected to dipping in water and in yeast water suspension for all treatment times. The raw samples subjected to PEF pre-treatment and subsequent dipping (PW5, PW15 and PY5, PY15) showed similar and lower moisture values than those treated only in dipping (W5, W15 and Y5, Y15). This result is probably explained by the increase of mass transfers from the sample to the dipping solution due to the modification of the cell membrane permeability induced by the PEF treatment. Similarly, all PEF pre-treated samples in the fried potato chips had a lower moisture content than the only dipping pre-treated ones. The moisture value of all fried potatoes subjected only to dipping pre-treatment was not substantially different from the untreated ones (C). Nonetheless, when compared to the raw samples, the moisture reduction due to frying was similar between the samples, ranging from 95 to 96%. This, along with the low standard deviation data, demonstrates the uniformity of the frying process across samples and repetitions.

Regarding the oil content, all treatments (W, Y, PW and PY) promoted an increase of oil uptake (between 34 and 41%) compared to the fried untreated sample (C). This increase is probably due to the slightly higher water content in the treated raw samples, especially for W and Y ones. In fact, during frying, the evaporation of the water leads to an absorption of frying oil (Aguilera & Gloria-Hernandez, 2000). However, in the PW and PY samples, subjected to PEF pre-treatment, which before frying showed the lowest water content compared to those subjected only to dipping (W and Y), the highest oil contents were found (38 and 41%). This result suggests that in the PEF-treated samples the absorption of oil does not depend only on the initial water content, but also on the induced tissue electroporation phenomenon. In the literature, there are conflicting results regarding oil absorption in potatoes after PEF pre-treatments. According to Fauster et al. (2018), Ignat et al. (2015) and Janositz et al. (2011), PEF treatment on potatoes led to a reduction in oil content of the final fried product in the shape of cubes or sticks (French fries). This result was ascribed to the transfer of water and intracellular substances to the potato surface due to the PEF-induced electroporation that, creating a barrier, reduced the oil uptake. This phenomenon is probably less evident in fried potato chips than in French fries. Furthermore, the frying phase in the current investigation was not modified as a function of the final reached moisture, whereas in the preceding work, the frying times

were shortened after PEF pre-treatment and thus allowed to obtain a reduction in the oil absorption.

3.1.4 Conclusions

The following conclusions can be derived from the findings of this research activity:

- the use of the yeast *A. pullulans* L1 strain in aqueous suspension (patented procedure No. WO2019058248A1) as pre-treatment of potato slices, was confirmed to be able to reduce AA on fried chips at dipping time longer than 5 min;
- the electroporation induced on raw potato slices by PEF-treatment led to the highest reduction of AA formation in potato chips at each tested dipping time (5 and 15 min);
- the effect of the PEF-treatment on the reduction of the AA generation in frying has been reduced when combined with yeast dipping for both tested treatment times;
- all the studied pre-treatments did not substantially influence the main final quality characteristics of potato chips.

The proposed strategies seem promising for the reduction of AA formation in fried potato products that will allow complying with the current Commission Regulation (EU) 2017/2158, without causing detrimental effects on their final quality. However, further optimizations of tested pre-treatments are needed for industrial applications. Particularly, the time needed for the metabolic response of yeast to PEF treatment has to be carefully considered to optimize the studied pre-treatments. Moreover, the effect of PEF on yeast activity should be further elucidated, in order to better exploit the yeast metabolism for AA reduction.

Chapter 4

Research outcomes: mitigation of acrylamide by control of heat treatment conditions

This chapter was based on **Paper III** and **Paper IV**:

Schouten M.A.; Tappi S.; Angeloni S.; Cortese M.; Caprioli G.; Vittori S.; Romani S. (2021). Acrylamide formation and antioxidant activity in coffee during roasting - A systematic study. *Food Chemistry*, 343, 1 May 2021, 128514, pp. 1-9 (Available online 1 November 2020, doi.org/10.1016/j.foodchem.2020.128514).

Schouten M.A.; Tappi S.; Glicerina V.; Rocculi P.; Angeloni S.; Cortese M.; Caprioli G.; Vittori S.; Romani S. (2021). Formation of acrylamide in biscuits during baking under different heat transfer conditions. *LWT – Food Science and Technology*, 153, 112541, pp. 1-8 (Available online 27 September 2021, doi.org/10.1016/j.lwt.2021.112541).

4.1 Effect of roasting degree in coffee

4.1.1 Introduction and aim of the research activity

Coffee is one of the most popular beverages in the world. Green coffee beans undergo a variety of changes during the roasting process as a result of various thermal reactions, most of them in the context of Maillard reactions (e.g. caramelisation, Strecker degradation, pyrolysis, etc.), that lead to the development of the desired physicochemical and sensory properties of the derived roasted coffee beverages, such as flavour, aroma and colour, but also to the formation of undesirable AA (Aguiar et al., 2016). Regarding AA levels, according to Commission Regulation (EU) 2017/2158, for “roast coffee”, food business operators should apply mitigation measures to ensure a minimum formation below the new reference level of 400 µg/kg (European Commission, 2017). Due to legislative restrictions and global consumption of coffee beverages, much research has been carried out to find possible solutions to reduce AA throughout coffee processing (Anese, 2016; **Paper I**).

One of the strategies for the control of AA level in coffee is the selection of high-quality green coffee beans. *Coffea arabica* (Arabica) is the most important coffee species for the processing industry, with about 60% of the total production, followed by *Coffea canephora* (Robusta). At the same roasting conditions, Robusta specie presents higher AA levels than Arabica, due to its higher content of asparagine in green beans (Bagdonaite et al., 2008; Summa et al., 2007). The roasting process conditions are strictly related to the formation of AA in coffee; the applied roasting degree, which can range from “light” to “dark” depending on time and temperature conditions adopted, seems to be a key factor (**Paper I**). Generally, the roasting degree is determined by the habit and consumers’ preferences in different countries (Anese, 2016). Several researchers stated that in the first stage of roasting the formation rate of AA reaches its maximum and decreases toward the end of the process, due to the high temperature and prolonged times (Bagdonaite et al., 2008; Bertuzzi et al., 2020; Budryn et al., 2015; Summa et al., 2007). However, most of these scientific studies are lacking important information concerning the roasting condition adopted, time-temperature profiles during the process, the number of replicates of roasting process and analysis, the main physicochemical and nutritional proprieties of the final roasted coffee (**Paper I**).

It is known that, despite the presence of AA, coffee is also a rich source of biologically active compounds with significant antioxidant proprieties (Summa et al., 2007). The effect

of roasting on the antioxidant activity of coffee has been extensively studied, but sometimes discordant results have been obtained. Many studies have found an increase in the antioxidant capacity in medium roasted coffee and a decrease in dark roasted one (Hečimović et al., 2011; Perrone et al., 2012; Vignoli et al., 2011, 2014); in contrast, other experimental studies have demonstrated a decrease of antioxidant capacity in light roasted coffee and an increase in dark roasted one (Daglia et al., 2000; Wen et al., 2005); further researchers have found an increase (Pokorná et al., 2015; Sánchez-González et al., 2005) or a decrease of antioxidant activity during roasting (Budryn et al., 2015; Pokorná et al., 2015; Summa et al., 2007). Characteristic coffee compounds linked to antioxidant activity are caffeine, trigonelline, chlorogenic and nicotinic acids; the content of which is highly influenced by the roasting process (Caprioli et al., 2014; Farah & Donangelo, 2006; Komes & Bušić, 2014; Vignoli et al., 2011; Zhou et al., 2012). Several health benefits are attributed to these compounds and their role in the prevention of chronic diseases such as cancer and cardiovascular pathologies have been the subject of a large number of scientific research (Aguiar et al., 2016).

The present research activity aimed to develop a comprehensive and systematic study on the formation of both AA and antioxidant activity in Arabica and Robusta coffee samples during the roasting process conducted under different time-temperature conditions. As a result, it should be possible to assess how the heat treatment can be directly linked to the presence/formation of unhealthy compounds, such as AA and healthy compounds, among which trigonelline, nicotinic and caffeoylquinic acids.

4.1.2 Materials and methods

4.1.2.1 Coffee sample preparation

Two green coffee (G) samples, belonging to *Coffea arabica* L. (Brazil) and *Coffea canephora* var. Robusta (India) both wet-processed, were supplied by the company ESSSE Caffè S.p.A. (Anzola dell'Emilia, Italy). The raw coffee beans were stored in a cold room at a temperature of 4 °C ± 1 and constant humidity until roasting.

Raw coffee beans batches of 250 g/run were roasted in a hot air pilot plant roaster with a rotating drum mod. EXPO 500/E (STA Impianti, Crespellano, Italy), pre-heated at 160 ± 2 °C. Coffee samples were roasted at five different roasting degrees: light (L), light-medium (LM), medium (M), medium-dark (MD) and dark (D). The air temperature inside the drum was measured, every 10 s, by the electronic control panel of the roaster in order

to monitor and assess the thermal profile of each cycle. The adopted roasting process conditions, in terms of final temperature and time, according to each coffee variety are reported in **Table 4.1**. The roasting conditions were chosen based on preliminary tests in accordance with the physicochemical characteristics of the two varieties analysed.

Table 4.1 - Times and temperatures applied to Arabica and Robusta coffee samples to obtain different degrees of roasting (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

Roasting degree:	L	LM	M	MD	D
Temperature (°C):	147	160	172	182	191
Arabica					
Time (min):	6	9	11	13	15
Robusta					
Time (min):	7	10	13	15	18

After roasting, the coffee samples were left to cool at room temperature, then transferred to a sealed glass jar and stored at 4 °C until analysis. A part of each green and roasted sample was ground using an electric grinder mod. M20 (IKA-WERKE, Staufen, Germany). Green coffee samples have been ground using a small amount of material in multiple cycles to avoid excessive heating of the product and to obtain a final homogeneous granulometry. Three repetitions were carried out for all roasting conditions, producing a total of 30 roasted coffee samples (5 × 3 for Arabica and 5 × 3 for Robusta), plus three replicates of both green ones.

4.1.2.2 Main quality parameters analysis

For each coffee sample, in order to assess the degree of roasting, the following analytical parameters were evaluated:

- weight loss (%) of coffee beans, determined as the percentage of weight variation between whole coffee beans before and after each roasting run;
- moisture (%) of green and roasted coffee, determined at 105 °C on ground samples by a gravimetric method as described in section 3.1.2.4;
- water activity (a_w) of green and roasted coffee, determined on ground samples using a dew-point hygrometer AQUALAB (Meter 4TE, Pullman, WA, USA) at 25 °C;
- density (g/mL) of green and roasted coffee beans, evaluated by volume displacement in a pycnometer using glycerine (ρ = 1.26 g/mL);

- colour of green and roasted coffee beans, measured by using a tristimulus spectrophotocolorimeter mod. ColorFlex EZ (HunterLab, Sunset Hills Road Reston, VA, USA) with geometry 45°/0°, illuminant D65 (6500 K) and equipped with a glass sample cup (64 mm diameter) and a 19.1 mm diameter measuring head. The instrument was calibrated with a white tile and black glass before the measurements. Colour was expressed in standard CIE L*a*b* scale and a* and b* parameters were converted into hue angle ($h° = (\tan^{-1}(b^*/a^*)/2\pi)\cdot 360$) (McGuire, 1992).

All analytical determinations were conducted 9 times for each green and differently roasted sample (e.g. 3 × 3 roasting repetitions).

4.1.2.3 Analytes extraction

For all analytes, water has been chosen as extraction solvent with a sample/solvent ratio of 1:10 since all monitored compounds were sufficiently polar for migrating and dissolving in water as reported in other works (Andrzejewski et al., 2004; Nielsen et al., 2006; **Paper II**). For AA extraction and sample purification, a previous procedure was followed (Andrzejewski et al., 2004) with some variations. Exactly, 1 g of ground coffee was spiked with 0.4 mL of AA-d_3 internal standard (500 ng/mL), then diluted with 9.6 mL of Milli-Q water. The extraction of monitored molecules was performed at 80 °C for 30 min, under magnetic stirring. The sample was then centrifuged for 10 min at 5000 rpm (3661 g) and the supernatant was collected and kept at 4 °C until use.

4.1.2.4 Asparagine, reducing sugars and acrylamide quantification

The analysis of asparagine, sugars (i.e. glucose, fructose and sucrose) and AA have been developed taking the cue from a previously developed procedure (**Paper II**). The present method has been implemented by using isotopically labelled internal standard (ILIS) and adding the analysis of sucrose. All AA precursors such as asparagine, sucrose, glucose and fructose were monitored in green coffee while AA in green and roasted beans.

Briefly, for the analysis of asparagine and reducing sugars, an aliquot of the supernatant was centrifuged at 5000 rpm for 10 min and diluted 1:20 with mobile phase while for sucrose analysis the dilution was 1:100. Then, before HPLC-MS/MS injection the diluted samples were filtered by a 0.2 µm syringeless filter. For AA analysis, the supernatant collected after water extraction was filtered by 0.45 µm syringeless filter and purified by SPE, following the previous procedure of Andrzejewski et al. (2004) adopting Oasis HLB

(Waters, Milford, MA, USA) and Bond Elut-Accucat (Agilent Technology, Santa Clara, CA, USA) cartridges. In brief, Oasis HLB columns were first conditioned with 3.5 mL of methanol (MeOH) and then with 3.5 mL of water. 1.5 mL of filtered supernatant was loaded onto cartridge and the sample was allowed to pass completely through the sorbent material and was followed with 0.5 mL of water. For AA elution, 1.5 mL of water was added onto the cartridge and the eluent was collected in an 8 mL glass vial. Before conditioning the second SPE column, a mark was placed on the outside of the cartridge at a height equivalent to 1 mL of liquid above the sorbent bed. The Bond Elut-Accucat column was conditioned with 2.5 mL of MeOH followed by 2.5 mL of water. The solvents used for conditioning were discarded. The eluent collected from the first cartridge was added to the Bond Elut-Accucat cartridge. The sample was allowed to eluate from the column up to the mark previously placed on the outside; the eluent was then collected to a 6 mL glass vial. Before injection into HPLC-MS/MS system, the collected samples have been filtered by 0.2 µm syringeless filter, discarding the first 0.5 mL to avoid collecting residual water used to wash the SPE cartridge, which could dilute any AA collected.

As previously reported in section 3.1.2.3 (**Paper II**), an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an ESI source operating in positive ionization mode and Kinetex Hilic analytical column (2.6 µm, 100 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA) preceded by a KrudKatcher ULTRA HPLC In-Line Filter (2.0 µm Depth Filter × 0.004 in ID) were used for HPLC-MS/MS experiments. The HPLC-MS/MS parameters of sucrose and AA-d_3 were optimized in FIA (1 µL of a 10 mg/L individual standard solution) (Agilent Technology, Santa Clara, CA, USA) and the other HPLC-MS/MS parameters were applied according to the previously described method (section 3.1.2.3, **Paper II**). For AA quantification the response factor was measured by calculating the ratio between the area of AA and AA-d_3. HPLC-MS/MS acquisition parameters of the MRM mode adopted for the quantification of AA-d_3 and sucrose were: precursor ion of 75 m/z and of 365 m/z, product ion 58 m/z and 365 m/z, fragmentor of 45 V and 130 V, collision energy of 8 V and 0 V, respectively. The LOQ and LOD have been calculated as 10:1 and 3:1 signal-to-noise ratio (SNR), respectively; the LOQ was 5 µg/L, while the LOD was 1 µg/L. The recovery was 90 ± 5% and the matrix effect was 110 ± 5%. All chemicals and reagents were of analytical grade. Results were expressed as µg/kg for AA and mg/kg for AA precursors on dry matter basis. For each analyte, three extractions and measurements were made for each sample type (e.g. 1 × 3 roasting repetitions).

4.1.2.5 Caffeine, trigonelline, chlorogenic and nicotinic acids quantification

The quantification of caffeine, trigonelline, nicotinic acid and chlorogenic acids was performed by following two developed procedures (Caprioli et al., 2013, 2014). Briefly, the supernatant collected after water extraction was diluted 1:20 in the mobile phase, filtered with 0.45 µm syringeless filter and then injected into a high-performance liquid chromatography-variable wavelength detector (HPLC-VWD) system. All analytes were monitored in green and roasted coffee except caffeine which was quantified only in green beans. The system used for the analysis was a Hewlett Packard (Palo Alto, CA, USA) HP-1090 Series II, made of an autosampler and a binary solvent pump. The separation of caffeine, trigonelline and nicotinic acid was achieved on a Gemini C18 110 Å analytical column (5 µm, 250 × 3 mm) from Phenomenex (Chesire, UK) using a mobile phase composed of water (A) containing 0.3% of formic acid and methanol (B), at a flow rate of 0.4 mL/min. The gradient program was: 0 min, 25% B; 0-10 min, 60% B; 10-15 min, 60% B; 15-20 min, 25% B; held at 25% until the end of the run at 25 min. The acquisition was performed at 265 nm for trigonelline and nicotinic acid and 270 nm for caffeine in the same run. The separation of chlorogenic acids was performed on a Polar-RP 80 Å analytical column (4 µm, 150 × 4.6 mm) from Phenomenex (Chesire, UK) with a mobile phase constituted by water (A) and methanol (B) both with 0.1% of formic acid, at a flow rate of 1 mL/min. The elution was carried out in gradient mode: 0-5.5 min, 25% B; 5.5-8 min, 50% B; 8-13.5 min, 50% B; 13.5-18 min, 25% B. The acquisition was performed by monitoring a wavelength of 325 nm for all three chlorogenic acids. All chemicals and reagents were of analytical grade. Results were expressed as mg/kg on dry matter basis. For each analyte, three extractions and measurements were made for each sample type (e.g. 1 × 3 roasting repetitions).

4.1.2.6 Antioxidant activity analysis

Antioxidant activity analysis was determined in green and roasted coffee extracts prepared according to the procedure described by Herawati et al. (2019). Around 2.5 g of powdered coffee was brewed with 50 mL of hot water at 95 °C for 1 min using a magnetic stirrer, cooled in an ice bath for 2 min and filtered with a 1300/80 125 mm filter paper (FILTER-LAB, Barcelona, Spain) and stored at –80 °C until the determinations.

To adequately represent the antioxidant activity of coffee samples, different in vitro methods were used: Folin-Ciocâlteu (FC) for the determination of total phenolic content, Ferric Reducing Antioxidant Power (FRAP) to evaluate the ability to reduce iron, ABTS

(2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays for representing the radical scavenging ability.

FC method was applied according to the procedure reported by Vignoli et al. (2011). In brief, 100 µL of the coffee extract was added to 300 µL of FC reagent (0.9 mol/L) and 1 mL of Na_2CO_3 solution (20% w/w); distilled water was then added until 10 mL was reached. The solution obtained was kept in the dark and at room temperature for 60 min. The total reducing capacity of the coffee samples was determined by measuring the absorbance at a wavelength of 765 nm with a UV-Vis spectrophotometer mod. UV-1601 (SHIMADZU EUROPA GmbH, Duisburg, Germany). The results were expressed in mg equivalent of gallic acid/100 g of ground coffee.

FRAP method was used following the procedure described by Sánchez-González et al. (2005). In brief, the FRAP reagent was obtained by combining 2.5 mL of TPTZ solution (10 mM) in HCl (40 mM), 2.5 mL of FeCl3·6H2O (20 mM) and 25 mL of acetate buffer (0.3 mM) at pH 3.6. The mixture obtained was warmed at 37 °C for 20 min in a stove mod. UF110 (Memmert, Schwabach, Germany). Subsequently, 900 µL of FRAP reagent, 90 µL of distilled water and 10 µL of diluted coffee extract were mixed. After 20 min at 37 °C, the absorbance was measured at a wavelength of 595 nm with a UV-Vis spectrophotometer mod. UV-1601 (SHIMADZU EUROPA GmbH, Duisburg, Germany). The results were expressed in mg equivalent of Trolox/100 g of ground coffee.

ABTS method was conducted according to Sánchez-González et al. (2005). Briefly, the ABTS radical cation solution was obtained by reaction of a stock solution of ABTS (7 mM) with potassium persulphate (2.45 mM), left to rest in the dark and at room temperature for 12-16 h. The ABTS radical cation solution was diluted with an alkaline phosphate buffer (pH 7.5, 5 mM) to reach an absorbance of 0.70 ± 0.02 at 734 nm. In 4 mL of obtained solution and 10 µL of coffee extract were added, then the absorbance was measured at 734 nm after 6 min using a UV-Vis spectrophotometer. The results were expressed in mg equivalent of Trolox/100 g of ground coffee.

DPPH method was used following the protocol of Vignoli et al. (2011). In brief, 50, 25, 20, 15 and 10 mg/mL concentrations for each coffee sample were prepared. A solution was prepared by mixing 0.5 mL of ethanolic DPPH solution (250 M), 1 mL of acetate buffer (100 mM; pH 5.5), 1 mL of ethanol and 10 µL of the sample at the different concentrations. After resting the solution for 10 min in the dark at room temperature, the absorbance was read at 517 nm using a UV-Vis spectrophotometer. The results were expressed in IC50 (coffee concentration able to reduce the radical DPPH by 50%) calculating the percentage

of inhibition of absorbance (IA%) for each coffee concentration. All chemicals and reagents were of analytical grade. All coffee extractions and antioxidants activity analyses were carried out 9 times for each green and differently roasted sample (e.g. 3 × 3 roasting repetitions).

4.1.2.7 Data analysis

The results were reported as mean value ± standard deviation and were processed as reported in section 3.1.2.5 of Chapter 3. In addition, the Pearson correlation coefficient (*r*), with a level of significance $p < 0.05$, was used to evaluate the relationship between the average values of AA, antioxidant activity (determined with FC, FRAP, ABTS and DPPH methods), total chlorogenic acids, trigonelline and nicotinic acid measured in all coffee samples.

4.1.3 Results and discussion

4.1.3.1 Roasting profiles and physicochemical characterization

The obtained time-temperature profiles as the average value of triplicate roasting cycles for each coffee sample are shown in **Figure 4.1**. As a result of the green beans insertion, a quick drop in the air temperature within the roaster (set at 160 °C) of roughly 70 °C was noted at the start of each roasting cycle. After 1 min the temperature started to rise, reaching the final value set for each roasting degree in both Arabica and Robusta samples. The Robusta sample took a longer time to achieve the final temperature set for each roasting degree than the Arabica sample. Due to their differences in composition, volume and bean shape, these two coffee types do not acquire a comparable degree of roasting at the same time (Romani, Cevoli, et al., 2012). The overlapping of heat profiles indicates that the roasting cycles presented a very high reproducibility.

Table 4.2 presents the results of the main roasting parameters analysed in all green and roasted coffee samples. As expected, coffee beans showed a significant and progressive weight loss, that at the longest roasting time (dark degree) reached around 17% and 18% in Arabica and Robusta samples, respectively. The weight loss in the first roasting degrees (from L to M) can be attributed to water loss, while from the MD degree it is mainly related to thermal degradation of organic matter into volatile compounds (C. L. Fernandes et al., 2019; Schenker & Rothgeb, 2017). In fact, the decrease in moisture (%) and a_w was faster in the early stages of the roasting process and then, from MD degree, became slower and similar in both Arabica and Robusta samples.

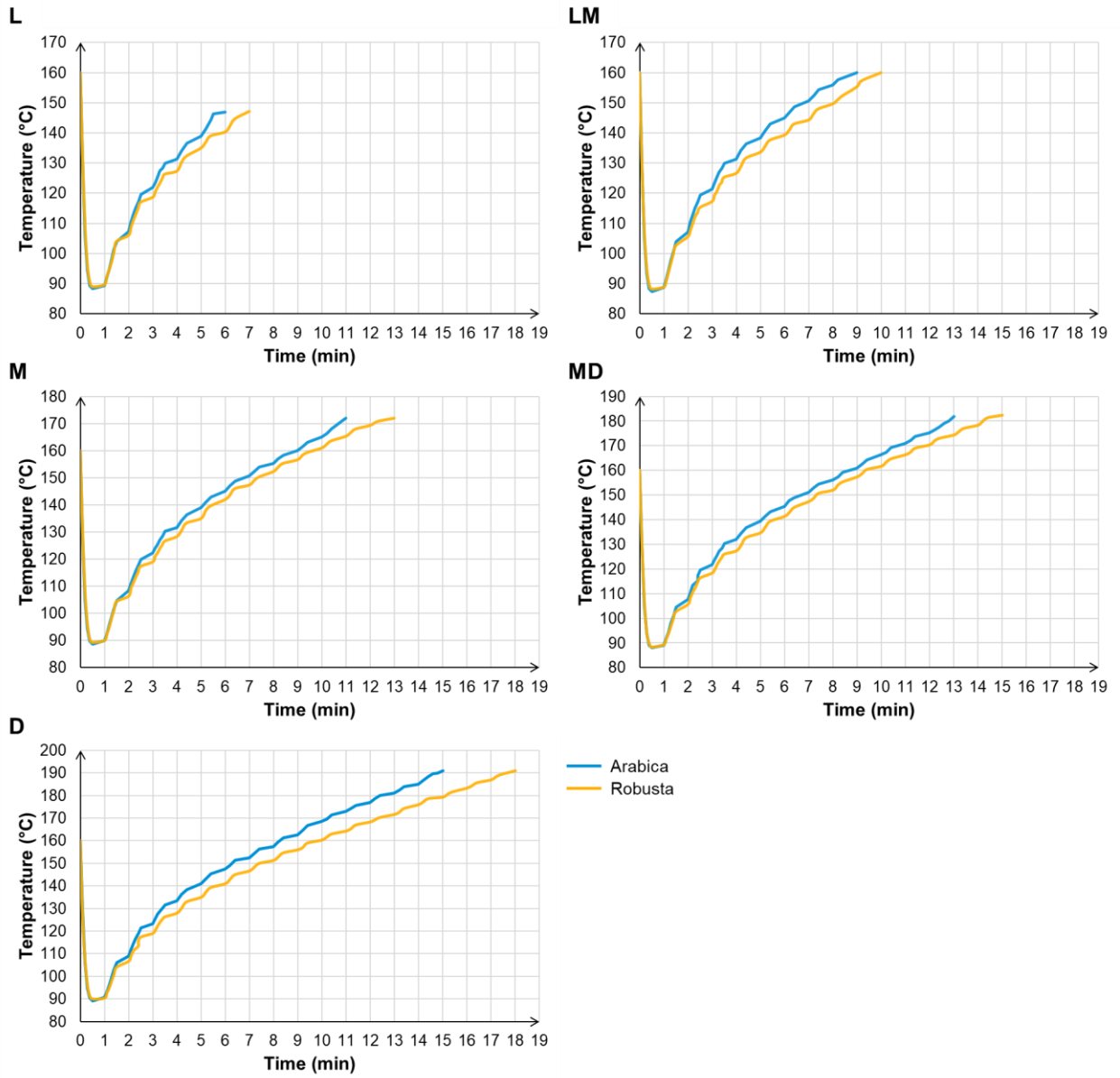


Figure 4.1 - Average time-temperature profiles recorded during the roasting of Arabica and Robusta coffee at L, LM, M, MD and D degrees (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

The moisture content in the Robusta sample until LM degree, was about 19% higher than that of Arabica, probably due to its higher initial moisture content in green coffee.

Another important roasting parameter is the density of coffee beans that decreased during roasting due to the simultaneous decrease in weight and increase in volume, associated with loss of water and generation of volatile compounds (Schenker & Rothgeb, 2017). The density values of the dark roasted Arabica and Robusta coffee samples were half those of the green samples for both Arabica and Robusta.

Table 4.2 - Roasting parameters of green (G) and differently roasted Arabica and Robusta coffee samples (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

Roasting degree	Weight loss (%)	Moisture (%)	Water activity (a_w)	Density (g/cm^3)	Lightness (L*)	Hue angle (h°)
Arabica						
G	--	9.4 ± 0.1^{b}	0.53 ± 0.00^{b}	1.1 ± 0.0^{a}	46.8 ± 1.1^{a}	82.8 ± 1.3^{a}
L	7.1 ± 0.1^{l}	4.2 ± 0.2^{d}	0.32 ± 0.02^{c}	0.8 ± 0.0^{b}	44.4 ± 1.4^{ab}	67.1 ± 0.9^{d}
LM	9.6 ± 0.1^{h}	2.8 ± 0.2^{f}	0.20 ± 0.01^{d}	0.8 ± 0.0^{c}	34.3 ± 2.5^{e}	62.2 ± 1.9^{f}
M	12.6 ± 0.1^{f}	1.7 ± 0.3^{gh}	0.12 ± 0.02^{e}	0.7 ± 0.0^{d}	28.2 ± 1.0^{f}	58.7 ± 1.1^{g}
MD	15.1 ± 0.1^{d}	1.1 ± 0.3^{h}	0.08 ± 0.03^{f}	0.6 ± 0.0^{ef}	23.3 ± 1.5^{g}	54.2 ± 1.6^{h}
D	16.8 ± 0.1^{b}	1.2 ± 0.2^{h}	0.07 ± 0.01^{f}	0.5 ± 0.0^{f}	20.6 ± 0.8^{h}	51.0 ± 1.4^{i}
Robusta						
G	--	11.6 ± 0.1^{a}	0.61 ± 0.01^{a}	1.2 ± 0.0^{a}	41.1 ± 1.3^{c}	78.0 ± 1.0^{b}
L	8.1 ± 0.1^{i}	5.1 ± 0.4^{c}	0.33 ± 0.01^{c}	0.8 ± 0.0^{b}	43.3 ± 1.5^{bc}	69.7 ± 0.7^{c}
LM	11.0 ± 0.3^{g}	3.4 ± 0.5^{e}	0.20 ± 0.02^{d}	0.8 ± 0.0^{c}	36.0 ± 1.0^{d}	65.4 ± 1.2^{e}
M	14.0 ± 0.3^{e}	2.1 ± 0.2^{g}	0.11 ± 0.01^{e}	0.7 ± 0.0^{d}	29.0 ± 1.2^{f}	60.7 ± 1.2^{g}
MD	15.9 ± 0.2^{c}	1.5 ± 0.1^{h}	0.08 ± 0.01^{f}	0.6 ± 0.0^{e}	24.1 ± 1.0^{g}	56.5 ± 1.4^{h}
D	18.0 ± 0.1^{a}	1.4 ± 0.2^{h}	0.07 ± 0.01^{f}	0.6 ± 0.0^{f}	21.2 ± 1.1^{h}	53.1 ± 2.0^{i}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Figure 4.2 - Visual appearance of green (G) and differently roasted Arabica and Robusta coffee samples (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

A substantial change in the colour of the coffee beans happened during roasting. As expected, the colour of both samples becomes increasingly brownish and more uniform at the greatest roasting degree as shown by the variations of lightness (L*) and hue angle

(h°). With the progression of Maillard reactions, the colour of coffee beans changed from greenish-grey-blue, typical of green coffee, to gradually yellow, orange, brown and brown-black in the dark roasting degree, as a result mainly of brown polymers formation (F. Fernandes, 2019). This colour change was also appreciated by the visual appearance of the coffee samples, as shown in **Figure 4.2**.

The roasting parameter values measured in the coffee samples are within usual ranges for the defined roasting degree. The M roasted coffee samples showed characteristics suitable for the preparation of an American-style drip coffee brew, while the D roasted coffee samples for the preparation of an Italian-style espresso coffee brew (Romani et al., 2003).

4.1.3.2 Influence of roasting degree on acrylamide content

The behaviours of AA formation in Arabica and Robusta coffee samples at various roasting degrees are presented in **Figure 4.3**. In green coffee samples, AA levels were always below the limit of quantification (LOQ) and increased during roasting at high temperatures. At the applied roasting conditions, the maximum AA levels were found in both Arabica and Robusta samples at the LM roasting degree, with a value of 731.3 ± 33.9 µg/kg for Arabica and 1128.3 ± 13.9 µg/kg for Robusta. Starting from the highest value (LM degree), the AA content decreased rapidly by 84.6% and 88.0% respectively for Arabica and Robusta D roasted samples, as the roasting degree was increased, achieving a similar final content. In addition, from M roasting degree the samples showed AA contents below the benchmark level of 400 µg/kg, reported in the EU Regulation 2017/2158 (European Commission, 2017). The general trend obtained in both samples during roasting confirmed, as reported in numerous studies, that AA formation is dominant during the first period of roasting and decreases toward the intensification of the thermal process (Bagdonaite et al., 2008; Bertuzzi et al., 2020; Esposito et al., 2020; Summa et al., 2007). However, only a few studies have attempted to identify a possible mechanism for AA evaporation or degradation after extended roasting (Badoud et al., 2020; Pastoriza et al., 2012). Further investigations are still required to identify the entire elimination mechanisms and to clarify if the degradation of AA contributes to the possible development of other toxic compounds, which may have a negative impact on human health. Comparing the two varieties of coffee analysed, Robusta coffee showed a significantly higher AA content than Arabica ones, especially at the lowest roasting

degrees. This is probably attributed to the different content of AA precursors in green coffee samples.

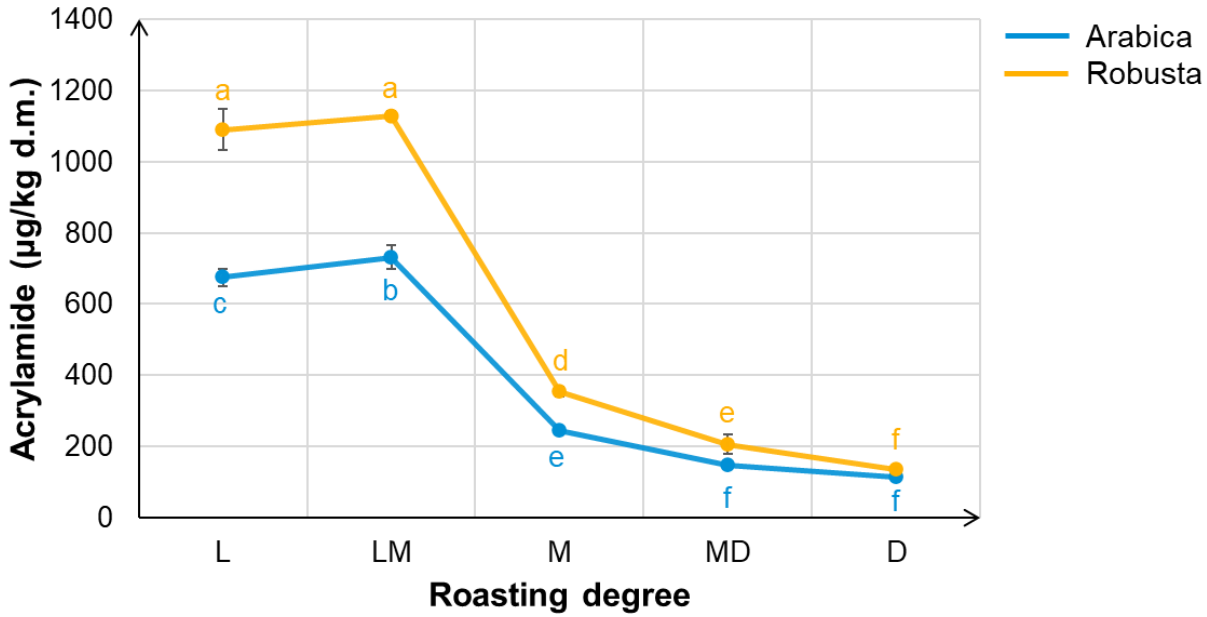


Figure 4.3 - Acrylamide levels (µg/kg) expressed on dry matter (d.m.) basis of Arabica and Robusta coffee samples roasted at various degrees (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark). Different letters indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The sum of total sugars was significantly higher in the Arabica green coffee beans than in Robusta (sucrose: 55630.3 ± 3601.2 mg/kg and 48013.7 ± 476.7 mg/kg respectively in Arabica and Robusta; reducing sugars: 12847.5 ± 155.5 mg/kg and 7996.0 ± 109.3 mg/kg respectively in Arabica and Robusta), while the levels of asparagine were 542.2 ± 41.5 mg/kg in Arabica and 803.0 ± 49.9 mg/kg in Robusta. The differences between Arabica and Robusta coffee in terms of AA content and AA precursors found in this study are in agreement with previous findings (Bagdonaite et al., 2008; Bertuzzi et al., 2020; Esposito et al., 2020; Summa et al., 2007).

4.1.3.3 Influence of roasting degree on antioxidant properties

The amount of trigonelline, nicotinic acid, caffeoylquinic acids and the antioxidant activity by reducing and radical scavenging abilities were tested to investigate if the applied roasting conditions altered the concentration and type of antioxidant components in the

studied coffee species. The contents of trigonelline, nicotinic acid and caffeoylquinic acids (3-CQA, 5-CQA, 3,5-diCQA) are reported in **Table 4.3**.

Table 4.3 - Chlorogenic acids (3-CQA, 5-CQA, 3,5-diCQA), trigonelline and nicotinic acid levels expressed on dry matter (d.m.) basis of green (G) and differently roasted Arabica and Robusta coffee samples (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

Roasting degree	3-CQA (mg/kg d.m.)	5-CQA (mg/kg d.m.)	3,5-diCQA (mg/kg d.m.)	Trigonelline (mg/kg d.m.)	Nicotinic acid (mg/kg d.m.)
Arabica					
G	4197.1 ± 24.9^{d}	24943.5 ± 111.7^{a}	2321.1 ± 9.6^{cd}	13540.1 ± 204.8^{a}	243.8 ± 32.8^{bcd}
L	6608.2 ± 650.4^{a}	20887.3 ± 2230.0^{b}	1811.4 ± 90.5^{e}	12902.5 ± 362.1^{a}	120.8 ± 0.8^{ef}
LM	5694.0 ± 490.2^{ab}	13356.9 ± 1021.5^{c}	1403.0 ± 144.8^{fg}	11443.2 ± 842.4^{b}	106-5 ± 2.5^{f}
M	4459.2 ± 259.9^{cd}	9346.6 ± 707.7^{de}	1093.1 ± 85.8^{gh}	10751.4 ± 67.5^{b}	191.4 ± 12.4^{def}
MD	2854.0 ± 310.7^{e}	5207.4 ± 667.5^{fg}	958.1 ± 4.0^{h}	7824.3 ± 217.9^{cd}	169.6 ± 22.9^{cde}
D	1977.0 ± 83.4^{e}	2940.3 ± 154.9^{g}	698.1 ± 13.8^{i}	4803.9 ± 376.7^{e}	267.9 ± 2.0^{bc}
Robusta					
G	2838.2 ± 4.4^{e}	21020.7 ± 33.3^{b}	2998.7 ± 106.6^{ab}	7093.8 ± 93.9^{d}	264.5 ± 18.1^{bcd}
L	6314.2 ± 379.8^{a}	22484.8 ± 1339.7^{ab}	3282.9 ± 253.4^{a}	8868.2 ± 381.4^{c}	128.2 ± 17.1^{ef}
LM	6602.8 ± 191.8^{a}	16591.9 ± 681.2^{c}	2683.1 ± 97.8^{bc}	8664.5 ± 188.4^{c}	126.5 ± 14.4^{ef}
M	5375.0 ± 515.6^{bc}	11750.3 ± 1365.3^{cd}	2021.7 ± 211.9^{de}	8837.3 ± 554.6^{c}	245.6 ± 28.9^{bcd}
MD	3815.5 ± 169.8^{d}	7606.9 ± 373.9^{ef}	1705.5 ± 39.9^{ef}	7157.3 ± 178.6^{d}	302.6 ± 10.8^{b}
D	2171.4 ± 178.0^{e}	3375.4 ± 375.6^{g}	1069.8 ± 183.7^{gh}	4407.8 ± 398.8^{e}	477.0 ± 13.2^{a}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Chlorogenic acids (CGAs) are the main phenolic antioxidant compounds in coffee and are formed by the esterification of quinic and hydroxycinnamic acids (Komes & Bušić, 2014). The major class of CGAs in coffee are caffeoylquinic acids (CQAs) and dicaffeoylquinic acids (diCQAs) with their main isomers 3-O-caffeoylquinic acid (3-CQA), 5-O-caffeoylquinic acid (5-CQA) and 3,5-O-di-caffeoylquinic acid (3,5-diCQA) (Farah & Donangelo, 2006; Komes & Bušić, 2014). The most abundant CGAs in the analysed coffee samples, both for Arabica and Robusta, were 5-CQA (about 80%), followed by 3-CQA and 3,5-diCQA. For Arabica (31461.7 ± 128.0 mg/kg) and Robusta (32081.9 ± 1972.3 mg/kg), the overall amount of the tested CGAs was higher in green and L roasted samples. The increased value in the L sample compared to the G one in Robusta coffee can probably be explained by the loss of other heat-sensitive compounds, which resulted

in a fictitious increase in the levels of the remaining ones. Moreover, an increase of 3-CQA from G to L roasting degree and a decrease or similar level of 5-CQA have been noticed as a possible result of the isomerization phenomenon of CGAs, that takes place at the beginning of the roasting process (Farah et al., 2005). In both species, the total amount gradually decreased as roasting time increased. Starting from their highest values, 5-CQA, 3-CQA and 3,5-diCQA were found to be reduced by about 90%, 70% and 70% in the D roasted samples, respectively. These results were attributed to their instability at high roasting temperatures, in fact, these phenolic substances are partially degraded during roasting and can be found in the pigment fraction as free quinic acid and as low molecular weight phenolic compounds (Vignoli et al., 2014). In addition, at the beginning of the roasting process, parts of the CGAs are incorporated into large molecular weight molecules generated through Maillard reactions forming several derived compounds (e.g. melanoidins) also characterized by antioxidant properties (Hečimović et al., 2011; Komes & Bušić, 2014). However, increasing roasting time leads to the degradation of melanoidins (Vignoli et al., 2014).

Trigonelline is another one of the major components of green coffee beans (Komes & Bušić, 2014). It is an alkaloid that is known to contribute to the development of desired volatile and non-volatile compounds, which are significant precursors to coffee flavour and aroma, as well as nutritional products (Farah et al., 2019). For example, trigonelline seems to possess some beneficial effects on diabetes or its complications and on the central nervous system which are related to its antioxidant activity as well (Zhou et al., 2012). Nonetheless, the contribution of trigonelline and its derivates to global coffee quality and health is mostly uncertain and requires further in-depth investigation (Farah et al., 2019). The trigonelline concentration of green coffee samples in both species was in good accord with the literature ranges (Farah et al., 2019), with greater values in Arabica coffee, and steadily declined during roasting, as displayed in **Table 4.3**. However, while in Arabica coffee a significant reduction was already observed in the LM samples, in Robusta one a first increase was observed in the L sample compared to the G one followed by a significant reduction observed in the MD and D samples. In D samples a reduction of about 60% for Arabica and 40% for Robusta was reached. Although the initial difference in trigonelline levels between the two coffee species, values were significantly similar in both MD and D samples, probably due to differences in cell wall resistance throughout the roasting process. Despite the reduction during roasting, the trigonelline

content in the ranges found in this study can still be considered relevant in terms of potential health benefits (Farah et al., 2019).

The major component derived from the thermal conversion of trigonelline during roasting is nicotinic acid. However, its content, at the D roasting degree compared to G samples, has increased only by 10% for Arabica coffee and 40% for Robusta one. These percentages confirm that trigonelline degradation leads also to the generation of other nitrogenous compounds including nicotinamide, N-methylpiridinium, 1,2-, 1,3-, 1,4-dimethylpiridinium (non-volatile compounds), pyridine and pyrrole derivates (volatile compounds) (Ashihara et al., 2015; Komes & Bušić, 2014).

In addition to the analysis of the main antioxidant compounds, antioxidant activity was assessed to have a better understanding of the health aspects of the examined coffee samples. Sánchez-González et al. (2005) reported that the FC, FRAP, ABTS and DPPH assays are effective methodologies for evaluating the antioxidant activity of coffee and coffee-based products. However, every essay tests a different mechanism for antioxidant activity, hence, with the aim of better representing this property, all mentioned determinations were used.

There were no significant differences in reducing and radical scavenging activity values between Robusta and Arabica green samples, as shown in **Table 4.4**. However, after roasting, the antioxidant activity of Robusta samples was considerably higher than that of Arabica samples at each roasting degree. The higher antioxidant activity of roasted Robusta coffee is ascribable to its higher caffeine (alkaloid with antioxidant proprieties) content whose levels are not significantly altered during roasting (Vignoli et al., 2014). The caffeine content of the analysed green coffee beans was significantly higher in Robusta (26519.7 ± 29.6 mg/kg) than in Arabica samples (23744.6 ± 85.8 mg/kg), while total CGAs and trigonelline contents were higher in Arabica. Hence, it can be assumed that a different combination of all singular components has led to the similar measured antioxidant activity in the two green coffee species.

During the first roasting minutes (from L to LM and M degrees), both reducing (FC and FRAP assays) and radical scavenging (ABTS and DPPH assays) capacities of coffee samples increased rapidly compared to green ones, in the range of 40-60% and 50-70% for Arabica and Robusta samples, respectively. After a plateau observed generally for all coffee samples, a modest decline in reducing and radical scavenging activities was observed for both species with longer roasting periods (MD and D degrees), however, this was not always significant. These outcomes are in agreement with earlier works in

which an increase in coffee antioxidant capacity at a light and medium roasting degrees and a subsequent decrease with the increasing of roasting time was observed (Bobková et al., 2020; Hečimović et al., 2011).

Table 4.4 - Reducing capacity (FC, FRAP) and radical scavenging activity (ABTS, DPPH) of green (G) and differently roasted Arabica and Robusta coffee samples (L: light; LM: light-medium; M: medium; MD: medium-dark; D: dark).

Roasting degree	FC (mg gallic acid/100 g)	FRAP (mg trolox/100 g)	ABTS (mg trolox/100 g)	DPPH IC_{50} (mg/mL) *
Arabica				
G	2402.6 ± 76.8^{e}	5026.1 ± 269.1^{f}	3693.1 ± 227.7^{f}	41.7 ± 3.2^{a}
L	5889.5 ± 863.3^{b}	8493.2 ± 876.0^{e}	5504.9 ± 249.5^{e}	23.7 ± 1.2^{bc}
LM	5771.2 ± 512.5^{b}	10172.4 ± 662.5^{de}	6101.9 ± 198.1^{de}	23.0 ± 1.1^{bc}
M	4475.4 ± 259.8^{d}	10172.4 ± 293.1^{de}	6771.0 ± 344.2^{cd}	21.8 ± 1.2^{cd}
MD	4281.4 ± 517.7^{d}	9803.9 ± 283.0^{de}	7008.1 ± 276.4^{c}	21.2 ± 1.0^{cd}
D	3103.6 ± 498.9^{e}	8529.5 ± 325.5^{e}	6676.0 ± 79.1^{cd}	27.4 ± 1.6^{b}
Robusta				
G	2390.7 ± 193.5^{e}	4253.6 ± 418.9^{f}	4396.4 ± 411.3^{f}	40.5 ± 2.7^{a}
L	6955.5 ± 903.9^{a}	13037.4 ± 1865.6^{ab}	8152.5 ± 911.5^{b}	14.4 ± 3.8^{f}
LM	6941.8 ± 1053.3^{a}	14444.6 ± 1215.2^{a}	9085.2 ± 318.1^{a}	17.1 ± 2.5^{def}
M	6741.9 ± 1342.7^{a}	13265.9 ± 625.3^{ab}	9079.8 ± 257.0^{a}	17.5 ± 1.9^{de}
MD	5297.9 ± 491.3^{bc}	12148.9 ± 687.1^{bc}	8912.4 ± 116.4^{a}	16.5 ± 1.8^{e}
D	4576.6 ± 288.4^{cd}	10709.1 ± 1453.4^{cd}	7996.8 ± 688.8^{b}	22.3 ± 0.7^{c}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).
* IC_{50} represents the concentration of coffee able to inhibit 50% of the radical solution, low values correspond to high antioxidant activity and vice versa.

The variation of antioxidant activity is related to a balance between the degradation and the neo-formation of antioxidant compounds. The highest antioxidant activity of L and/or M roasted coffee can be attributed to the release of low molecular weight phenols from the green coffee constituents and to the formation of compounds by Maillard reactions during the roasting process (Komes & Bušić, 2014; Vignoli et al., 2014). In specific, various antioxidant mechanisms have been attributed to melanoidins, such as chain breakage, metal chelation, radical scavenging and reducing abilities (Delgado-Andrade et al., 2005). The majority of melanoidins are generated early in the roasting process and their relative contribution to the overall antioxidant activity increases as the roasting

temperature rises, mainly due to the degradation of CGAs during the thermal process (Smrke et al., 2013). The overall decrease in antioxidant activity observed in this study in the last roasting stages might indicates that the degradation of antioxidant compounds is not fully compensated by the production of new ones.

4.1.3.4 Correlation of coffee acrylamide content and antioxidant activity

The results of Pearson's correlation matrix analysis carried out between the values of AA, chlorogenic acids, trigonelline, nicotinic acid, reducing and radical scavenging capacities identified in Arabica and Robusta coffee samples are provided in **Table 4.5**.

A strong correlation was found between the reducing capacity, measured by FC and FRAP methods, and the radical scavenging ability determined by ABTS and DPPH assays. Because the antioxidant activity in the DPPH assay was expressed in IC_{50}, low IC_{50} values correspond to higher antioxidant activity values and vice versa, the correlation of all used methods with DPPH method was negative. The results of antioxidant activity determined by FC, FRAP and DPPH methods were also significantly correlated with the AA content results. Both AA and antioxidant activity increased remarkedly during the early roasting degrees indicating a strong relationship between Maillard reactions and the formation of antioxidant compounds. The following decrease is observed for both AA and antioxidant activity but to a different extent. Indeed, AA levels dropped by more than 80%, while for antioxidant activities the decrease was lower. This is evidenced by the fact that the AA content was unrelated to the antioxidant activity assessed by the ABTS assay, probably because ABTS results decreased during roasting in both coffee samples slower than data measured by the other methods. In detail, the reduction percentage of reducing and radical scavenging capacities in coffee samples, calculated between the reached maximum value (L or LM degree) and the D roasted degree were 50% and 30%, 20% and 30%, 30% and 50%, 5% and 10% respectively for Arabica and Robusta samples determined by the FC, FRAP, DPPH and ABTS methods. The varying percentages of reduction of the antioxidant activity outlined that the trend of these coffee health attributes was influenced by both the coffee composition and the analytical procedures utilized for the determination. The trigonelline concentration in both species was positively connected with CQAs, showing gradual degradation of both classes of components during roasting, and negatively correlated with nicotinic acid, indicating an inverse relationship between them (**Table 4.5**).

Table 4.5 - Correlation matrix of acrylamide, reducing capacity (FC, FRAP), radical scavenging activity (ABTS, DPPH), chlorogenic acids (total CQAs), trigonelline and nicotinic acid values of Arabica and Robusta coffee samples roasted at all roasting degrees investigated.

	Acrylamide	**FC**	**FRAP**	**ABTS**	**DPPH**	**CQAs**	**Trigonelline**
FC	0.83	-	-	-	-	-	-
FRAP	0.65	0.88	-	-	-	-	-
ABTS	0.42	0.74	0.95	-	-	-	-
DPPH	–0.62	–0.87	–0.94	–0.89	-	-	-
CQAs	0.48	0.20	-0.15	–0.35	0.21	-	-
Trigonelline	0.26	0.12	-0.20	–0.46	0.20	0.69	-
Nicotinic acid	–0.65	–0.39	–0.11	0.15	0.15	–0.60	–0.66

Pearson correlation coefficient (r): $0.6 \leq r \leq 1$ = positive linear correlation, $-1 \leq r \leq -0.6$ = negative linear correlation and $-0.6 < r < 0.6$ = no correlation.

4.1.4 Conclusions

The following conclusions can be derived from the findings of this systematic research activity:

- the roasting process adopted confirmed a decrease in both antioxidant activity and AA content; however, the thermal reduction observed in medium, medium-dark and dark roasted Arabica and Robusta samples was greater for AA than for antioxidant activity, which was only slightly reduced;
- under the roasting conditions, samples of coffee roasted from medium to dark degrees showed AA concentrations below than the reference value given in the Commission Regulation (EU) 2017/2158 (400 g/kg);
- the importance of considering the impact of heat treatments on both toxic (AA) and beneficial compounds (e.g. CGAs, trigonelline, nicotinic acid), applying a holistic risk-benefit research approach, was emphasised.

Any changes in coffee roasting conditions, as well as species selection with the intention of reducing AA in the product, could also lead to some reduction in the final content of beneficial compounds, such as antioxidants and biologically active compounds. Furthermore, the overall results obtained, such as those from other scientific comprehensive studies, may be important and useful for both the food industry and

international authorities to identify and evaluate potential interventions to reduce AA formation in the most at-risk widely consumed food products.

4.2 Effect of baking heat-transfer mode in biscuits

4.2.1 Introduction and aim of the research activity

Bakery products, along with fried potatoes and roasted coffee, are one of the food categories at risk of AA due to the presence of asparagine and reducing sugars in the most used ingredients, as well as the baking process carried out at temperatures above 120 °C, which triggers the Maillard reaction (Mesías et al., 2016). According to Commission Regulation (EU) 2017/2158, for "biscuits and wafers", food business operators should apply mitigation measures to ensure a minimum AA formation below the benchmark level of 350 µg/kg (European Commission, 2017).

Baking is a complicated process that involves simultaneous heat and mass transfers, resulting in a temperature gradient that affects the rates of chemical reactions in the food matrix (Mesías et al., 2016; Mesías, Delgado-Andrade, et al., 2020). Several studies on bakery products have found that the formation of AA takes longer at low temperatures, whereas at high temperatures the maximum level is reached in a short time (Özge Çetinkaya Açar & Gökmen, 2010; Ahrné et al., 2007; Amrein et al., 2004; Elmore et al., 2005; Mogol & Gökmen, 2014; Sakin-Yilmazer et al., 2013). On the other hand, the mode of heat transfer could also influence AA development by causing differences in the formation of temperature gradients within the product, which, in turn, influences the rate of chemical reactions, including process-induced AA formation (Anese et al., 2008; Claus et al., 2008; Sakin-Yilmazer et al., 2013; Van Der Fels-Klerx et al., 2014). For example, a study of Claus et al. (2008) investigated the differences between the use of a multi-deck oven, characterized by heat transfer via conduction and radiation, and of a convection oven, based on forced air circulation, for bread baking. The results indicated that at 220 °C, AA levels were 60% higher in bread baked in a ventilated oven (173.9 µg/kg) than in a static oven (109.6 µg/kg), whereas at 260 °C these differences were reduced by up to 35%, probably due to the high formation rate of AA at this high temperature. The highest AA levels in bread baked in the convection oven were attributed to the forced air circulation, leading to faster and more intense drying of the bread surface crust. In addition to these conventional heat transfer modes, there are alternative baking methods

(e.g. dielectric, vacuum and steam heating), but it has been shown that these do not always achieve the same results in terms of the quality of some bakery products (Anese et al., 2008; Mogol & Gökmen, 2014; Sakin-Yilmazer et al., 2013).

The present research activity aimed to evaluate the influence of two commonly used baking methods, such as conduction and forced air convection, on the AA formation in biscuits. Moreover, AA precursors (i.e. asparagine, glucose and fructose) and some quality characteristics (i.e. weight loss, moisture, water activity, texture and colour) were analysed in biscuits during baking in order to identify the optimal baking conditions to obtain the lowest AA level and at the same time acceptable quality characteristics of the final product.

4.2.2 Materials and methods

4.2.2.1 Biscuits sample preparation

The biscuit dough was prepared in a standardised way following a basic formulation described by Canali et al. (2020). In detail, the ingredients purchased from a local market (Cesena, Italy), such as wheat flour (500 g), sucrose (125 g), pasteurized eggs (125 g), butter (100 g), milk (100 mL) and a leavening agent containing sodium diphosphate and carbonates (15 g), were weighed and placed in a household mixer mod. Bimby Robot TM31 (Vorwerk, Wuppertal, Germany). All the ingredients were mixed and kneaded with the speed regulator set to position 5 (approximately 3000 rpm) for 1 min and 30 s, with the direction of rotation reversed after 80 s to clean the walls of the robot's kneading container. The dough mix was then compacted by kneading it by hand for about 30 s. The moisture content and a_w of the dough, as prepared, were 23.53 ± 0.41% and 0.90 ± 0.01, respectively. After letting it rest in a refrigerator at 4.0 ± 0.1 °C for 20 min, the dough has been sheeted to a thickness of 3.0 mm by a pasta filler machine mod. SFSI 42040050T (GAM International, Santarcangelo di Romagna, Italy) and cut by using a stainless-steel circular mould of 6 cm diameter. A part of each raw dough sample was freeze-dried for reducing sugars, asparagine and AA analyses.

The uncooked biscuits were placed on a baking tray positioned on the centre level of a domestic electric oven mod. Procombi Plus (AEG-Electrolux, Berlin, Germany) and baked in static mode (S) with heat transfer mainly by conduction and vented mode (V) with forced air convection. In the forced convection condition, the hot air was constantly re-circulated inside the oven by a fan installed on the backside of the oven chamber

(airspeed: 0.5 m/s); while the fan was turned off in the static air condition. For both baking methods, the oven was preheated for the time necessary to reach and maintain a constant air temperature equal to 175 °C and the baking of the samples was carried out for 18, 20, 22, 24 and 26 min. The temperature and time conditions studied were selected after preliminary trials to obtain biscuit samples baked at different levels. For each baking condition, 3 batches of 10 biscuits (about 180 g of raw dough per batch) were produced. For each baking cycle, the temperature of the air inside the oven chamber and inside biscuits were recorded every 20 s using 1 mm diameter type K thermocouples (RS PRO Italy, Milan, Italy) with an accuracy of 0.1 °C, connected to the acquisition and registration system mod. 34970 (Agilent Technologies, Santa Clara, CA, USA). A thermocouple was placed in the centre of the oven chamber above the baking tray and another one inside a biscuit positioned in a central position of the baking tray. The temperatures were monitored continuously throughout the duration of each cooking cycle.

After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room temperature for 1 h.

4.2.2.2 Asparagine, reducing sugars and acrylamide quantification

The quantification of asparagine, sucrose, reducing sugars (fructose and glucose) and AA has been performed by following previous analytical methods (C. L. Fernandes et al., 2019; **Paper III**) with some adjustments especially in the extraction and purification steps. In detail, 1 g of ground biscuits was fortified with AA-d_3 (0.5 mL at 500 ng/mL) and, according to preliminary optimization trials, 9.5 mL of water was added into it. Then, the sample was agitated with a vortex mixer for 30 s and sonicated for 1 h at room temperature with an ultrasonic bath (FALC, Treviglio, Italy) at a frequency of 59 kHz. After centrifugation at 5000 rpm for 10 min, the sample was filtered with filter paper and the lipids were removed by a defatting process with hexane (four times with a total volume of 35 mL). After that, the sample was filtered with a 0.45 µm filter, and for the analysis of asparagine, glucose and fructose, it was diluted 1:40 in the mobile phase, filtered with a 0.2 µm pore membrane filter and injected into an HPLC-MS/MS system. An aliquot of sample was diluted 1:50000 in the mobile phase and injected into the HPLC-MS/MS apparatus for sucrose analysis. For the AA quantitation, a purification step composed of a double SPE has been carried out. With the purpose to increase the content of AA in the final sample extract, several volumes (1.5, 3, 4 and 6 mL) of the sample have been individually loaded in the first cartridge but keeping then the same elution volume. The

best results in terms of SNR of the quantitative transition of AA (1.5 mL, SNR = 8; 3 mL, SNR = 18; 4 mL, SNR = 26; 6 mL, SNR = 27) have been generated with 4 mL. The first employed cartridge was the Oasis HLB (Waters, Milford, MA, USA). This was first conditioned with 3.5 mL of MeOH and then with 3.5 mL of water. 4 mL of sample were loaded onto cartridge followed by 0.5 mL of water. The sample was allowed to pass completely through the sorbent material. For AA elution, 1.5 mL of water was added onto the cartridge and the eluent was collected in a 3 mL glass vial. Before conditioning the second SPE column, a mark was placed on the outside of the cartridge at a height equivalent to 1 mL of liquid above the sorbent bed. The Bond Elut-Accucat (Agilent Technology, Santa Clara, CA, USA) column was conditioned with 2.5 mL of methanol followed by 2.5 mL of water. The solvents used for conditioning were discarded and the eluent collected from the first cartridge was added to the Bond Elut-Accucat cartridge. The sample was allowed to eluate from the column up to the mark previously placed on the outside; the eluent was then collected into a 6 mL glass vial. Lastly, the samples were filtered with a 0.2 µm filter and injected into HPLC-MS/MS. The same procedure has been adopted for the extraction of the analytes from the freeze-dried dough. Also in this research activity, the instrument employed for the HPLC-MS/MS analysis was an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an ESI source operating in positive ionization mode and a Kinetex Hilic analytical column (2.6 µm, 100 mm × 4.6 mm, Phenomenex, Torrance, CA, USA) preceded by a KrudKatcher ULTRA HPLC In-Line Filter (2.0 µm Depth Filter × 0.004 in I.D.). The mobile phases were composed of water (A) and acetonitrile (B) both with 0.1% of formic acid and the separation has been obtained at 0.8 mL/min in gradient elution mode. The composition of the mobile phase varied as follows: 0-2.5 min, isocratic condition, 85% B; 2.5-3.5 min, 85-70% B; 3.5-5.5 min, isocratic condition, 70% B; 5.5-6.5 min, 70-60% B; 6.5-10 min, isocratic condition, 60% B; 10-12 min, 60-85% B; 12-20 min, isocratic condition, 85% B. The injection volume, the temperature of the drying gas in the ionization source, the gas flow, the nebulizer pressure and the capillary voltage were the same as previously described. The acquisition was performed in MRM mode and the most abundant transitions were used for quantification while the other for confirming the analyte. For AA quantification the response factor was measured by calculating the ratio between the area of native AA and AA-d_3. LOD and LOQ have been calculated by injecting gradually lower concentrations of standard mixtures and the concentration that determined an SNR ratio of 3 was assigned to LOD while that generated an SNR of 10

was assigned to LOQ. The present method was characterized by good sensitivity, which agrees with those reported in the literature (C. L. Fernandes et al., 2019; Wenzl et al., 2006; **Paper III**). LOQ for glucose, fructose, sucrose, asparagine and AA were 50, 50, 10, 5 and 5 µg/kg, respectively. All chemicals and reagents were of analytical grade. Results were expressed as µg/kg for AA, mg/kg for AA precursors and g/kg for sucrose on dry matter basis.

4.2.2.3 Main quality parameters analysis

For each biscuit sample, the following analytical determinations were carried out:

- weight loss (%) of biscuits, determined as the percentage of weight variation among 10 biscuits before and after each baking cycle;
- moisture (%) of raw dough and baked biscuits, determined by drying up to constant weight at 105 °C as reported in section 3.1.2.4;
- water activity (a_w) of raw dough and baked biscuits, determined according to section 4.1.2.2;
- colour of raw and baked biscuits, measured using a tristimulus spectrophotocolorimeter mod. ColorFlex EZ (HunterLab, Sunset Hills Road Reston, VA, USA) as previously reported in section 4.1.2.2. Colour was expressed in standard CIE L*a*b* scale and the chromatic parameters were converted into hue angle ($h° = (\tan^{-1}(b^*/a^*)/2\pi)\cdot 360$) (McGuire, 1992) and browning index ($BI = ([(X-0.31)\cdot 100])/0.17$, where $X = (a^*+1.79\cdot L^*)/(5.645\cdot L^*+a^*-3.012\cdot b^*)$) (Sakin-Yilmazer et al., 2013);
- texture of baked biscuits, performed at room temperature using a Texture Analyser mod. TA-HDi500 (Stable Micro System, Surrey, UK) equipped with 25 kg load cell and a three-point bending test holder and probe. The distance of the two beams was 30 mm and the other settings were: pre-test speed of 5.00 mm/s, test speed of 1.00 mm/s, post-test speed of 10.00 mm/s and distance of 5 mm. The downward movement was advanced till the biscuit was broken. The acquired parameters were expressed as hardness, calculated by means of maximum force (N) values; index of crispness, calculated by using the linear distance between the first and the last peaks registered (Tylewicz et al., 2019; **Paper II**) and fracturability, expressed as one/breakpoint distance between the origin of curve till the point where the biscuit breaks (Romani, Balestra, et al., 2012).

All analyses were performed in triplicate for each baking batch per sample, except for the determination of colour and texture, which were evaluated on both surfaces (upper and

lower) of 5 biscuits for each baking batch per sample and on 10 biscuits for each baking batch per sample, respectively.

4.2.2.4 Data analysis

The results were reported as mean value ± standard deviation and were processed as reported in section 3.1.2.5 of Chapter 3. The relationship between the average values of AA, the content of precursors and the physical-chemical characteristics of the biscuits baked under different conditions were evaluated with Pearson correlation coefficient (r). An r-value between $0.60 \leq r \leq 1.00$ indicates a positive linear correlation, $-1.00 \leq r \leq -0.60$ indicates a negative linear correlation and $-0.60 < r < 0.60$ indicates no correlation, with a significance level $p < 0.05$.

4.2.3 Results and discussion

4.2.3.1 Baking profiles

Figure 4.4 shows the air temperature inside the oven and the temperature of the biscuits during the baking process at 175 °C for 18, 20, 22, 24 and 26 min. Every temperature profile is the average of three baking cycles, indicating that the baking process was highly standardized.

Due to the opening of the oven to insert the biscuits tray, the air temperature inside the oven decreased rapidly and markedly from 175 °C to about 115 °C at the start of each baking process. Depending on the mode of heat transfer (static or ventilated), the temperature of the air inside the oven returned to the initial temperature of 175 °C in different ways during the baking of the biscuits. In detail, with the ventilated mode the oven reached the set temperature in the first two min, more quickly than with the static mode that required about 10 min. This is because in a static mode the heat comes from the electrical resistances located both at the top and bottom of the oven chamber (natural convection), while in the ventilated mode the hot air is distributed through a fan by forced convection, so the chamber heats up faster and more evenly (Marcotte, 2007; Sakin et al., 2009; Walker, 2016). In fact, it has been proven that using a fan in the oven compared to not applying a fan resulted in a higher heat transfer coefficient ($W/m^2 \cdot K$), one of the main parameters of the baking process time and efficiency together with the resulting product quality (Sakin et al., 2009). In both static and ventilated modes, it is necessary to consider that heat is also distributed across the contact area between products and the baking tray as well as transferred by radiation from the hot oven's inner walls (Cevoli et

al., 2020; Sakin et al., 2009). The total radiative heat transfer coefficient is relatively low and unaffected by the type of heat transfer mode, as it is primarily determined by the oven wall material and temperature, whereas the total convective heat transfer coefficient is more than twice as high under ventilated conditions as it is under static conditions without the fan (Sakin et al., 2009).

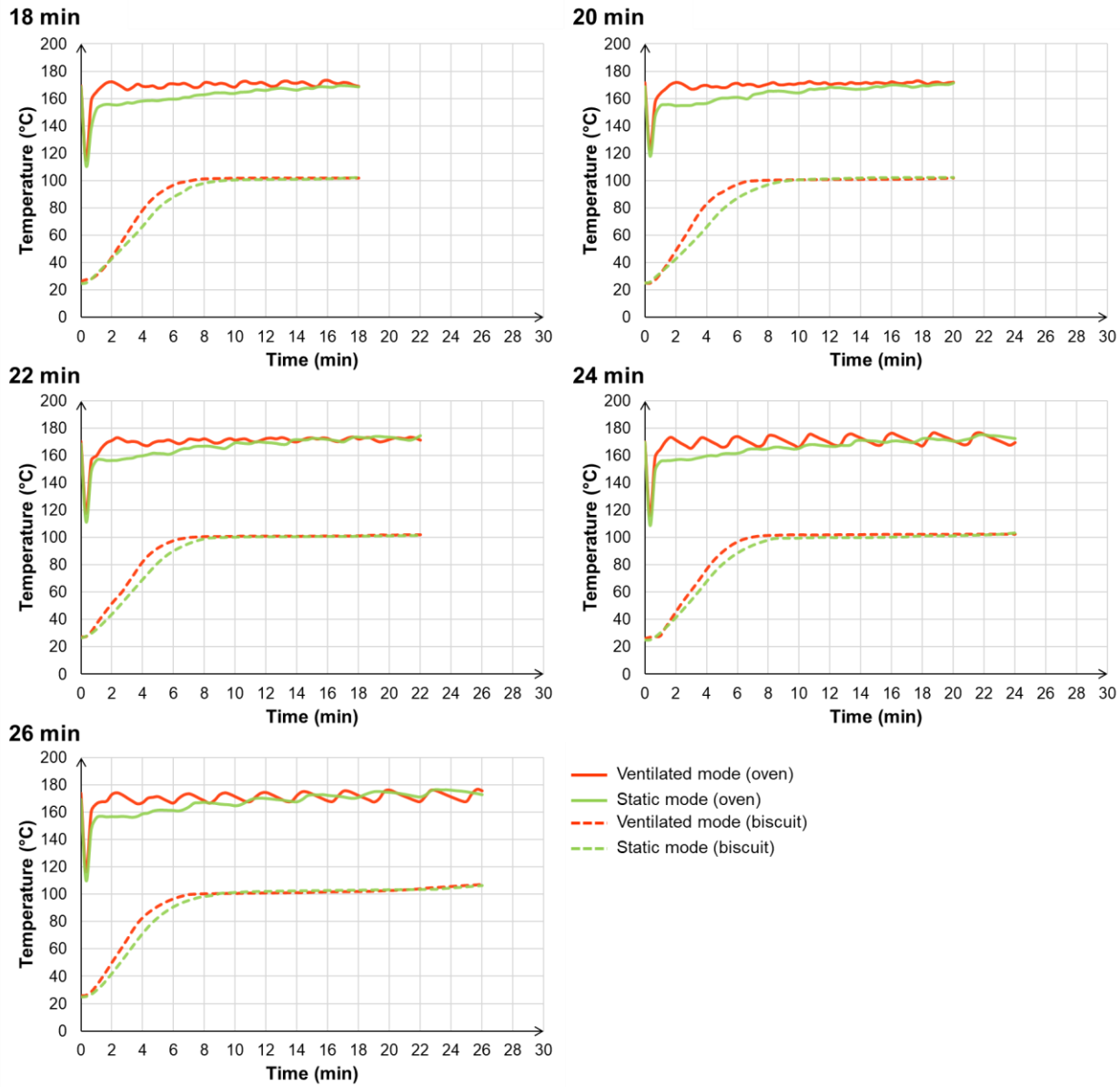


Figure 4.4 - Average time-temperature profiles of the oven chamber and of the inner part of biscuit samples recorded during the baking process in ventilated and static modes for 18, 20, 22, 24 and 26 min.

The thermal profiles of the biscuit during baking were influenced by the differences in temperature measured inside the oven set up at the two different baking modes, as

expected (**Figure 4.4**). In comparison to the biscuit baked in static mode, the inside of the biscuit baked in ventilated mode reached a temperature of 100 °C slightly faster (approximately 2 min earlier). Only after 26 min of baking, in both modes, the biscuits exceeded the temperature of 100 °C, indicating a start of over-baking. Generally, the time-temperature behaviour of food baked in the oven can be divided into three different phases: the first phase is characterised by a slight increase in temperature, followed by a faster increase up to 100 °C, which represents the second phase; finally, in the third phase the temperature of 100 °C is kept constant due to the evaporation of the water inside the matrix (Manley & Clark, 2011).

4.2.3.2 *Influence of heat-transfer method on acrylamide content*

The formation of AA and the levels of its precursors in the biscuits at the considered baking times were influenced by changes in the time-temperature profiles of S and V biscuit samples during the first minutes of baking.

In **Figure 4.5**, AA levels of biscuit samples during baking in ventilated and static modes for different times are shown. In detail, AA was not detected in the raw dough; after the first 18 min of baking the AA levels found in S and V samples were respectively of 130.0 ± 3.0 µg/kg and 153.1 ± 14.5 µg/kg without significant differences.

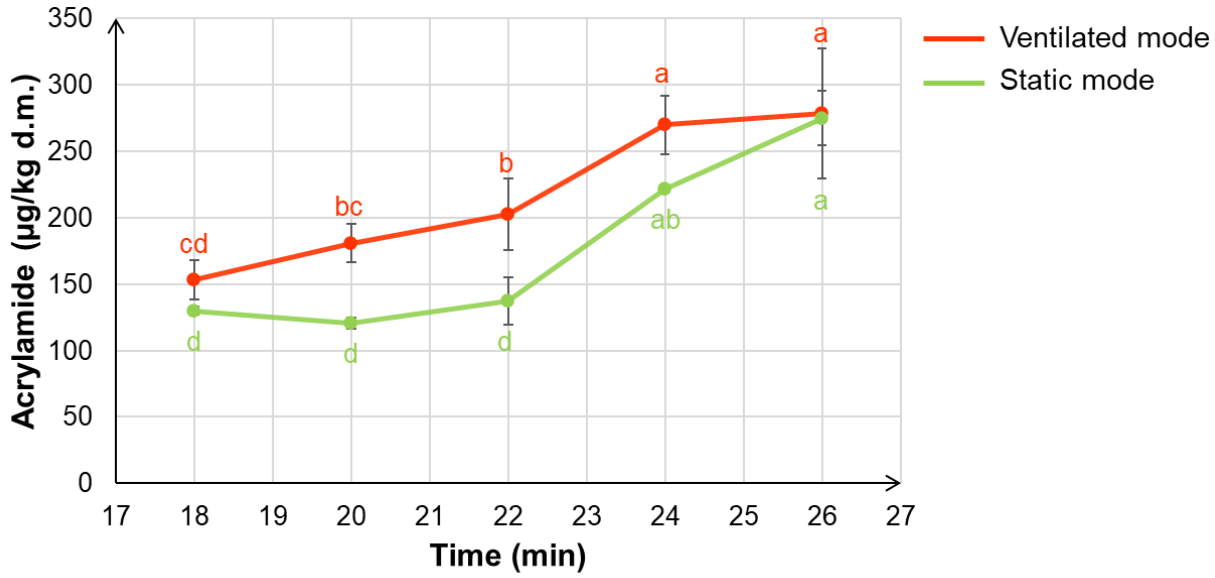


Figure 4.5 - Acrylamide (µg/kg) levels expressed on dry matter (d.m.) basis of biscuit samples during baking in ventilated and static modes at 175 °C for different times. Different letters indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

At longer baking times, the AA levels increased, reaching the highest values at 26 min of about 275.0 µg/kg for both biscuit samples. Several studies have found that increasing the baking time increases the amount of AA formed in biscuits (Amrein et al., 2004; Cheng et al., 2014; Courel et al., 2009; C. L. Fernandes et al., 2019; Mogol & Gökmen, 2014; Žilić et al., 2020). In a recent study, Žilić et al. (2020) baked biscuits at 180 °C for 7, 10 and 13 min in a ventilated oven confirming that the AA formation is proportional to the baking time; the authors found AA levels of 72.3 µg/kg and 861.7 µg/kg after 13 min of baking in biscuits formulated with refined wheat flour and hulless oat flour, respectively. The major differences in AA values between S and V biscuits were found after 20 and 22 min of baking; the samples baked with the ventilated mode showed significantly higher AA values compared to those baked with static mode (**Figure 4.5**). The higher AA values found in V biscuit samples can be related to the fact that in a ventilated mode the heat is distributed faster and more evenly during baking, favouring more dryness of the product and, as a result, increasing the AA formation.

The levels of asparagine, glucose, fructose and sucrose found in raw dough and in differently baked biscuits samples are reported in **Table 4.6**. All the AA precursors in both S and V biscuit samples decreased compared to the raw dough, indicating their involvement in the Maillard reaction and thus AA formation. The levels of asparagine, the major AA precursor in cereal products, observed in the biscuit samples during baking are consistent with those previously reported in the literature for biscuits baked under various time-temperature conditions (Nguyen et al., 2016; Wenzl et al., 2006). The concentrations of asparagine appeared to rise after 20 min of baking in both S and V biscuit samples, reaching concentrations of 42.2 ± 2.4 mg/kg and 38.7 ± 1.7 mg/kg, respectively. This increase could be the result of the thermal hydrolysis of the proteins in the flour (Žilić et al., 2020). The levels of asparagine in the S and V samples baked for 26 min were reduced by 64.8% and 56.9%, respectively, in successive baking times. As a result, it's possible that the variation in asparagine content was caused by both protein hydrolysis and its participation in chemical reactions leading to its partial degradation, such as the Maillard reaction (Curtis et al., 2014; Hamlet et al., 2008; Nguyen et al., 2017; Weiss et al., 2018; Žilić et al., 2020). This amino acid turns out to be the limiting precursor in baked products since its concentration is lower compared to the reducing sugars one, 60.1 ± 1.1 mg/kg versus 89.6 ± 3.1 mg/kg, respectively. Pearson's correlation analysis confirmed this, with a significant r-value of −0.90 indicating a strong association between asparagine content and AA value.

Table 4.6 - Levels of asparagine (mg/kg), glucose (mg/kg), fructose (mg/kg) and sucrose (g/kg) expressed on dry matter (d.m.) basis in the raw dough and in biscuits baked in static (S) and ventilated (V) modes for different times.

Baking time (min)	Asparagine (mg/kg d.m.)	Glucose (mg/kg d.m.)	Fructose (mg/kg d.m.)	Sucrose (g/kg d.m.)
Raw				
0	60.1 ± 1.1^{a}	34.3 ± 2.1^{a}	55.3 ± 4.3^{a}	209.3 ± 16.6^{a}
S				
18	29.1 ± 0.6^{c}	16.1 ± 2.1^{cde}	27.9 ± 3.9^{ef}	154.3 ± 19.9^{b}
20	42.2 ± 2.4^{b}	18.6 ± 0.2^{bcde}	48.3 ± 3.7^{abc}	156.9 ± 13.0^{b}
22	28.3 ± 1.1^{c}	16.4 ± 3.9^{cde}	45.2 ± 4.1^{bcd}	184.1 ± 8.6^{ab}
24	20.4 ± 1.4^{e}	15.0 ± 1.7^{de}	26.6 ± 3.6^{f}	162.5 ± 17.1^{ab}
26	14.8 ± 0.7^{f}	11.4 ± 1.2^{e}	36.5 ± 4.2^{cdef}	181.5 ± 8.5^{ab}
V				
18	25.9 ± 1.4^{cd}	14.8 ± 0.1^{de}	33.0 ± 5.9^{def}	142.3 ± 18.4^{b}
20	38.7 ± 1.7^{b}	23.8 ± 1.6^{bc}	48.6 ± 4.9^{abc}	181.6 ±16.3^{ab}
22	20.9 ± 3.5^{de}	20.3 ± 3.5^{bcd}	36.9 ± 1.1^{def}	165.0 ± 22.3^{ab}
24	21.4 ± 1.3^{de}	16.4 ± 1.1^{cde}	40.5 ± 4.2^{cd}	182.7 ± 19.6^{ab}
26	16.7 ± 2.8^{ef}	24.6 ± 6.1^{b}	55.8 ± 7.3^{a}	173.5 ± 1.3^{ab}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Except at 26 min for glucose and 24 and 26 min for fructose, no significant differences in the quantity of reducing sugars were identified between the two differently baked biscuit samples. These differences, however, were not reflected in the AA content, which did not correlate with glucose and fructose r-values of −0.54 and −0.24, respectively. Similar to asparagine, glucose and fructose contents follow a non-linear trend, probably due to simultaneous reduction in the Maillard reaction and formation through the hydrolysis of sucrose that occurs during long baking (Amrein et al., 2004; Hamzalıoğlu & Gökmen, 2020; Van Der Fels-Klerx et al., 2014). However, after 26 min of baking, the sucrose content of both samples S and V was slightly reduced non-significantly compared to the measured quantity in the raw biscuit dough (**Table 4.6**) and was unrelated to the AA content of the baked biscuit samples ($r = -0.21$). Some previous studies have found that low sucrose hydrolysis occurs under the usual baking conditions for biscuits (Gökmen et al., 2007; Graf et al., 2006). In fact, a very effective strategy suggested to control AA

content is the use of sucrose (non-reducing sugar) in the formulation of bakery products (Gökmen et al., 2007; Graf et al., 2006; Nguyen et al., 2016).

4.2.3.3 *Influence of heat-transfer method on main quality characteristics*

The evolution of the main quality parameters of biscuit samples, whose data are provided in **Table 4.7** and **Table 4.8**, confirmed that in a ventilated mode, heat is diffused faster and more evenly during baking, resulting in a higher AA formation.

Table 4.7 - Moisture (%), water activity (a_w), weight loss (%) and texture proprieties of biscuits baked in static (S) and ventilated (V) modes for different times.

Baking time (min)	Moisture (%)	Water activity (a_w)	Weight loss (%)	Hardness (N)	Crispness (linear distance)	Fracturability (1/mm)
S						
0	23.6 ± 0.4^{a}	0.91 ± 0.00^{a}	--	--	--	--
18	7.8 ± 0.1^{b}	0.58 ± 0.00^{b}	17.2 ± 0.0^{f}	44.7 ± 2.7^{f}	57.3 ± 1.5^{g}	0.4 ± 0.0^{e}
20	5.9 ± 0.2^{c}	0.47 ± 0.01^{c}	19.2 ± 0.0^{e}	45.8 ± 2.2^{f}	78.6 ± 2.5^{e}	0.6 ± 0.0^{d}
22	4.7 ± 0.2^{e}	0.39 ± 0.01^{e}	19.7 ± 0.2^{d}	63.9 ± 6.1^{c}	104.8 ± 6.8^{d}	0.6 ± 0.0^{c}
24	3.6 ± 0.2^{g}	0.28 ± 0.01^{g}	20.9 ± 0.1^{c}	62.6 ± 1.4^{c}	125.1 ± 2.1^{c}	0.7 ± 0.0^{ab}
26	2.8 ± 0.0^{h}	0.21 ± 0.00^{h}	21.3 ± 0.1^{bc}	71.3 ± 2.5^{b}	169.6 ± 3.9^{a}	0.7 ± 0.0^{a}
V						
0	23.5 ± 0.5^{a}	0.90 ± 0.01^{a}	--	--	--	--
18	7.5 ± 0.0^{b}	0.59 ± 0.01^{b}	17.5 ± 0.2^{f}	51.4 ± 0.5^{e}	67.8 ± 0.7^{f}	0.4 ± 0.0^{e}
20	5.4 ± 0.3^{d}	0.44 ± 0.02^{d}	19.2 ± 0.1^{e}	56.4 ± 0.6^{d}	104.2 ± 2.5^{d}	0.5 ± 0.0^{d}
22	4.3 ± 0.4^{f}	0.36 ± 0.05^{f}	19.8 ± 0.1^{d}	66.2 ± 1.2^{c}	123.4 ± 1.0^{c}	0.6 ± 0.0^{c}
24	3.5 ± 0.2^{g}	0.28 ± 0.03^{g}	21.6 ± 0.3^{b}	69.8 ± 2.5^{bc}	147.5 ± 2.1^{b}	0.6 ± 0.0^{cb}
26	2.9 ± 0.1^{h}	0.26 ± 0.02^{h}	22.3 ± 0.1^{a}	83.6 ± 3.5^{a}	169.0 ± 5.5^{a}	0.7 ± 0.0^{a}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The baking process promoted the dehydration of the biscuits; the moisture and the a_w for both biscuit samples progressively decreased. In particular, the moisture and a_w of S samples baked for 20 and 22 min were significantly higher than those of V samples. Several studies have reported that low moisture and a_w trigger the formation of AA, while, in systems containing residual water, the formation of AA is reduced because evaporation reduces the effective temperature, even in putatively dry areas of the product such as the

external portions (Bråthen & Knutsen, 2005; Esposito et al., 2020; Matthäus et al., 2004). The strong relationship between pathways formation and these influencing factors was confirmed by the correlation of AA content results with weight loss (r = 0.84), moisture (r = −0.85) and a_w (r = −0.93). Significant differences between S and V samples in terms of weight loss after baking were only evident after 24 and 26 min of baking when the samples baked in a ventilated mode showed a higher value due to greater water evaporation under forced air convection conditions.

Table 4.8 - Lightness (L*), hue angle (h°) and browning index (BI) of upper and lower surfaces of biscuits baked in static (S) and ventilated (V) mode for different times.

Baking time (min)	Lightness (L*)	Hue angle (h°)	Browning index (BI)	Lightness (L*) **	Hue angle (h°) **	Browning index (BI) **
S						
0	70.9 ± 0.1^{d}	82.9 ± 0.0^{a}	74.6 ± 0.2^{g}	70.9 ± 0.1^{a}	82.9 ± 0.0^{a}	74.6 ± 0.2^{d}
18	77.1 ± 0.3^{a}	76.3 ± 0.4^{b}	76.8 ± 2.93^{f}	64.6 ± 0.5^{b}	69.6 ± 0.2^{b}	114.6 ± 5.7^{c}
20	74.5 ± 0.3^{b}	74.8 ± 0.6^{c}	83.5 ± 2.6^{e}	63.6 ± 0.6^{bc}	69.3 ± 0.3^{c}	114.2 ± 5.4^{c}
22	71.4 ± 0.1^{c}	71.9 ± 0.3^{d}	89.3 ± 2.6^{d}	60.5 ± 0.3^{de}	68.0 ± 0.0^{e}	120.5 ± 5.1^{ab}
24	69.9 ± 0.2^{d}	70.8 ± 0.0^{ef}	90.9 ± 2.3^{cd}	59.4 ± 0.2^{de}	67.2 ± 0.2^{f}	120.6 ± 2.4^{ab}
26	67.0 ± 0.1^{e}	70.1 ± 0.1^{g}	95.0 ± 2.5^{ab}	58.8 ± 0.3^{e}	67.2 ± 0.2^{fg}	124.2 ± 4.4^{a}
V						
0	70.7 ± 0.2^{d}	82.9 ± 0.0^{a}	74.6 ± 0.1^{g}	70.7 ± 0.2^{a}	82.9 ± 0.0^{a}	74.6 ± 0.1^{d}
18	74.6 ± 0.3^{b}	74.2 ± 0.2^{c}	79.0 ± 2.4^{f}	63.2 ± 0.1^{bc}	68.5 ± 0.2^{d}	114.2 ± 2.5^{c}
20	70.0 ± 0.4^{d}	71.1 ± 0.1^{de}	91.7 ± 2.9^{cd}	60.8 ± 0.2^{bcde}	67.8 ± 0.2^{e}	117.5 ± 2.4^{bc}
22	68.7 ± 0.3^{e}	70.3 ± 0.2^{fg}	91.8 ± 2.7^{cd}	60.5 ± 0.3^{cd}	67.2± 0.1^{f}	118.9 ± 2.8^{ab}
24	67.9 ± 0.1^{e}	70.0 ± 0.1^{g}	94.1 ± 1.6^{ab}	59.8 ± 0.2^{de}	67.3 ± 0.2^{fg}	119.6 ± 2.7^{ab}
26	66.7 ± 0.3^{e}	69.6 ± 0.1^{g}	96.9 ± 1.9^{a}	59.5 ± 0.2^{de}	66.8 ± 0.1^{g}	119.5 ± 3.0^{ab}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).
** Lower surface of the biscuits.

The texture is another important quality parameter for biscuits and in general bakery products. Together with the formation of AA, hardness ($r = 0.93$), crispness ($r = 0.97$) and fracturability ($r = 0.90$) also increased progressively in all samples as the baking time increased. This positive correlation is attributed to the increase in the degree of baking of the biscuit and the lowering of the moisture content. The S biscuits samples showed

hardness and crispness values lower than V samples at all baking times, except for crispness at 26 min, in which values were not statistically different. The flow of heated air circulating throughout the oven in ventilated mode helps to accelerate the crust formation, especially on the surface of biscuits (Palazoğlu et al., 2015). On the other hand, the fracturability increased with prolonging baking times but did not show significant differences between samples baked with the two different modes.

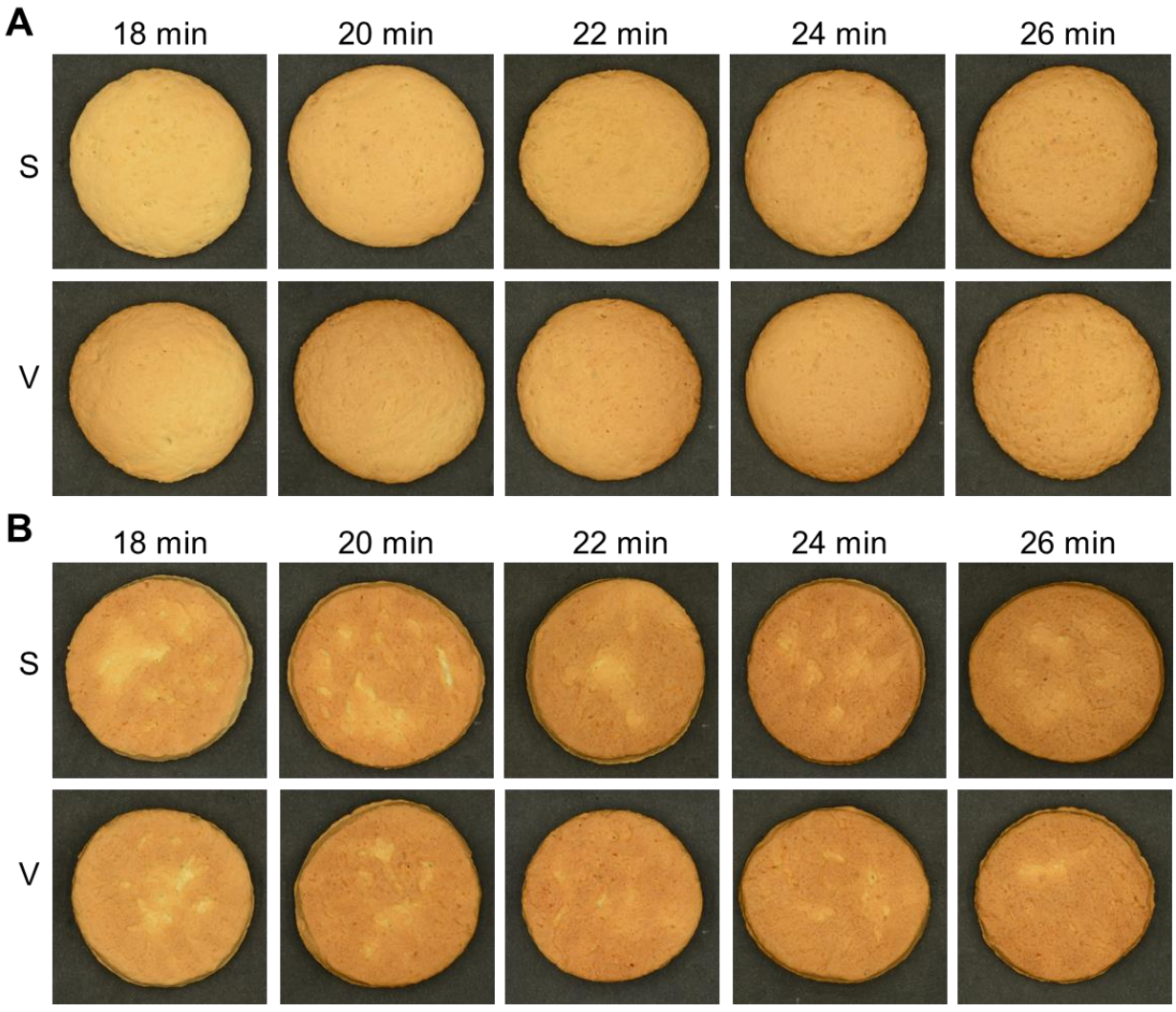


Figure 4.6 - Visual appearance of the upper (A) and lower (B) surface of biscuit samples baked in static (S) and ventilated (V) mode for different times.

During baking, there was also a significant change in the colour of the surface of the biscuits. As expected, as the level of AA increased, the colour of the biscuits became progressively more brownish prolonging the baking times on the upper/lower surfaces according to significant *r* correlation values of −0.90/−0.92/−0.82 and 0.82/0.88/0.81 for lightness, hue angle and browning index, respectively.

The lower surface of the biscuits had a greater browning index and a lower hue angle value in all samples, indicating a more intense colouration than the upper surface. The upper surface of samples baked in ventilated mode showed lower lightness and hue angle with a higher browning index value, thus a redder and browner colour than S samples (**Table 4.8**). No significant differences in upper surface colour between both samples were found after 26 min of baking. Regarding the colour of the lower surface of the biscuit, no peculiar differences were found between V and S samples except for the hue angle in the samples baked for 18, 20 and 22 min showing a slightly redder colouration for the biscuits baked in a ventilated oven. This is due to the fact that in both modes, the heat was mostly transmitted from the baking tray to the lower biscuit surface. The colour differences between static and ventilated biscuits were also appreciated by the visual appearance of the samples, as shown in **Figure 4.6**.

4.2.4 Conclusions

The following conclusions can be derived from the findings of this research activity:

- it has been confirmed that baking process carried out in an oven set up in ventilated mode the heat is distributed in a more homogeneous way causing a faster temperature rise in the product, which consequently dehydrates and bakes faster than in static mode;
- this was demonstrated by both the recorded time-temperature profiles and the results of the baking parameters (weight loss, moisture, a_w, texture and colour) measured on the biscuits over time. In particular, biscuits baked in a ventilated mode were crisper and darker in colour than those baked in a static mode;
- the heat transferred by convection in the ventilated mode promoted a higher formation of AA in the biscuits compared to those baked under static conditions, mainly after 20 and 22 min of baking;
- on the basis of the overall quality parameter results and the lowest AA levels reached, it can be concluded that the best baking conditions were in the static baking mode at 175 °C for 20 and 22 min;
- at the formulation and the baking conditions studied, all biscuit samples including the over-cooked ones, showed concentrations of AA lower compared to the reference value reported in Commission Regulation (EU) 2017/2158 (350 μg/kg).

Although the present research activity found that, under the applied formulation and baking conditions, biscuits are a low/medium risk product category in terms of AA content,

a deeper understanding and characterisation of the presence of this toxic and carcinogenic compound in this type of heat-processed product depending on the baking technology used is still needed. Biscuits are widely consumed in the population and may contribute greatly to the human dietary intake of AA. However, the levels in biscuits highly vary depending on their composition and baking conditions.

Chapter 5

Research outcomes: mitigation of acrylamide by the use of alternative ingredients

This chapter was based on **Paper VI** and **Paper VII**:

Schouten M.A.; Fryganas C.; Tappi S.; Romani S.; Fogliano V. (2022). The use of chickpea flour is an effective formulation strategy to reduce the acrylamide formation in biscuits. Manuscript submitted for publication (11 February 2022).

Schouten M.A.; Fryganas C.; Tappi S.; Romani S.; Fogliano V. (2022). The use of kidney bean flour with intact cell walls reduces the formation of acrylamide in biscuits. Manuscript submitted for publication (18 January 2022).

5.1 Effect of lupin and chickpea flour in biscuits

5.1.1 Introduction and aim of the research activity

In recent years there have been several studies investigating the effect of different flours on the formation of AA in bakery products as this ingredient is the main source of asparagine, the designed limiting factor for this type of food product (Miśkiewicz et al., 2012; Negoiță et al., 2017; Salazar et al., 2012; Sazesh & Goli, 2020; Žilić et al., 2020; **Paper V**). In detail, the complete or partial replacement of wheat flour in the bakery products formulations with alternative flour types (e.g. rye, oats, quinoa) or with flours with different extraction degrees, characterised by a high ash content, was evaluated in the literature (Ciesarová et al., 2014; Claus et al., 2006; Manolache et al., 2019; Mesías et al., 2016; Miśkiewicz et al., 2012; Sazesh & Goli, 2020). Cereal varieties with higher amounts of free asparagine have also been shown to result in biscuits with higher levels of AA (Žilić et al., 2017).

Asparagine levels in cereals depend on various internal and external factors such as genetics, growing conditions, harvest year, fertilization, milling and storage conditions (Curtis et al., 2018; Gökmen, 2015). Due to higher levels of asparagine in the outer layers of the grain, products made from whole grain result in higher AA levels despite other health promoting effects (Springer et al., 2003). However, Žilić et al. (2020) prepared biscuits from a variety of different flours (i.e. wheat, oat, rye, barley, triticale, maize) and observed that the asparagine levels in the flours did not exactly correlate to the AA levels that were measured in the biscuits. This means that the concentration of this limiting precursor does not exactly correspond to the concentration of AA in the final biscuits. Similar conclusions were made by Capuano et al. (2009) who toasted bread at different temperatures for different times. This observation might be an indicator that other flour compounds also have an impact on the Maillard reaction and, consequently, on AA formation.

For example, some studies have shown a protective effect against AA formation by proteins in the ingredients adopted in the formulation of different food products (Miśkiewicz et al., 2012, 2020; Rydberg et al., 2003; Tareke et al., 2002). Rydberg et al. (2003) studied the effect of protein-rich ingredients (i.e. cod meat) added to potato-based products observing a reduction in AA in the final products up to 70%. It has been hypothesised that this effect may result from covalent binding of proteins to the reactive side chains of amino groups by AA or from competitive reactions of proteins. Furthermore,

in a recent study of Miśkiewicz et al. (2020), chickpea proteins extract showed a mitigation effect of AA formation in a biscuit-like low-moisture model system. It was suggested that the observed 40% reduction of AA formation was due to the increased thermal stability of the reducing sugars by the chickpea protein extract. In the presence of chickpea protein extract, the carbohydrates presented a higher ordering of their crystallographic structures and this reduced their availability to react with asparagine and lead to AA formation (Miśkiewicz et al., 2020). On the other hand, legume flours are usually higher in fibre content than cereal flours. High fibre content flour from Okara (soya-based by-product), was shown to promote the AA formation by binding water and reducing the water activity of the dough which promotes the Maillard reaction (Palermo et al., 2012). From the currently published studies investigating the use of different flours, it is not possible to deduce if the differences in AA levels in the final products are solely based on the varying asparagine content or also related to structural properties due to the different fibre and protein contents in the flours.

The present research activity aimed to investigate the potential AA mitigating effects of the biscuit food matrix prepared with different flours by standardizing the initial asparagine content in the formulations. Biscuits were formulated by replacing 20, 40 and 60% of wheat flour with protein-rich legume flours from lupins and chickpeas. Asparagine was added proportionally to all formulations to have the same concentration in all biscuits. In this way, we were confident to evaluate the role of the other flours characteristics on AA formation. In addition to the chemical compositions, several structure-related effects on the formation of AA during baking were investigated, together with the impact on the colour and texture characteristics of the final products.

5.1.2 Materials and methods

5.1.2.1 Biscuit sample preparation

The biscuit doughs were formulated with 100% wheat flour (Wageningen, The Netherlands) and with the wheat flour partially replaced by 20, 40 and 60% lupin flour (Frank Food Products, Twello, The Netherlands) or chickpea flour (NutsinBulk, Dublin, Ireland). The sample codes according to their flour percentages are given in **Table 5.1**. The biscuit doughs with a similar asparagine content were prepared in a standardised way according to the basic recipe from the AACC method 10-54 (AACC, 2009) with the following proportion of baking ingredients purchased from local and online markets

(Wageningen, The Netherlands): total flour (250.0 g), sucrose (105.0 g), shortening (100.0 g), sodium chloride (3.13 g), sodium bicarbonate (2.5 g), ammonium bicarbonate (1.25 g), high-fructose corn syrup (3.75 g), non-fat dry milk (2.5 g), distilled water and asparagine. The amounts of distilled water and external asparagine added in each formulation were standardised to approximately 17% and 70 mg/kg, respectively, calculated from the moisture and asparagine content determined in the different flours and considering their percentages.

Table 5.1 - Codes of the biscuit samples and corresponding percentages of flours in formulation.

Sample code	Lupin flour (%)	Chickpea flour (%)	Wheat flour (%)
W	--	--	100
L20	20	--	80
L40	40	--	60
L60	60	--	40
C20	--	20	80
C40	--	40	60
C60	--	60	40

To assure a homogeneous distribution in the dough, asparagine, high-fructose corn syrup and sucrose were solubilized in water at room temperature for 1 min using Thermomix TM5 (Vorwerk, Wuppertal, Germany) by setting the speed regulator to position 2. Successively, the other dry ingredients and shortening were added and mixed thoroughly for 1 min by setting the speed regulator to position 5 and reversing the direction of rotation after 30 s. The dough was shortly kneaded by hand to compact it, wrapped in plastic foil and let to rest for 20 min in a refrigerator at 4 °C. For some subsequent analyses, parts of the raw dough samples were freeze-dried and finely ground with a mortar.

The dough was rolled out to a thickness of about 3 mm by a pasta filler machine (Marcato, Campodarsego, Italy) and cut by using a stainless-steel circular cup pastry of 6 cm diameter. For each formulation and baking batch, 8 biscuits were baked in an electrical oven mod. OV185C (Inventum, Arnhem, The Netherlands) with convection mode at 175 °C for 5, 7 and 9 min. The different baking conditions were chosen in preliminary tests in order to obtain biscuits that were neither too much raw nor overcooked. The biscuits were placed on a baking tray in the middle position inside the oven and for each baking cycle, the air temperature inside the oven chamber was recorded every 20 s using a digital

thermometer equipped with type K thermocouples mod. RS Pro 206-3722 (RS Components, Corby, UK) to ensure equal temperature exposure between the repetitions. After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room temperature for about 1 h. All biscuit formulations and baking times were performed in triplicate, resulting in a total of 24 biscuits for each sample and for each baking time.

5.1.2.2 Main quality parameters analysis

The following analytical determinations were carried out for the flour and biscuit samples characterization:

- moisture (%) of flours, raw doughs and biscuits, determined on ground samples by gravimetric method at 105 °C using an oven mod. Heraeus Series 6000 (Thermo Scientific, Berlin, Germany) as reported in section 3.1.2.4 of Chapter 3;
- water activity (a_w) of flours, raw doughs and biscuits, determined on ground samples using mod. LabMaster a_w-meter (Novasina AG, Lachen, Switzerland) at 25°C, setting the time and temperature factors stability at 2 min and 2 min, respectively;
- weight loss (%) of biscuits, determined as the percentage of weight variation among 8 biscuits before and after each baking cycle;
- total nitrogen content (%) of flours, determined using the Dumas method with mod, Flash EA 1112 Protein Analyser (Thermo Fisher Scientific, Waltham, MA, USA) and a conversion factor of 6.25;
- total fat content (%) of flours, determined by gravimetrical method with Soxhlet extraction of 5 g with 200 mL of petroleum ether at 60 °C for 3 h;
- total dietary fibre (%) of flours, established by an enzymatic-gravimetrical method according to AOAC Method 991.43 (AOAC, 2000), using a total dietary fibre assay kit (Megazyme, Illinois, Chicago, IL, USA);
- ash content (%), measured gravimetrically by weighing 1 g of flour in ceramic crucibles and incineration at 525 °C for 5 hours in a muffle furnace (Gallenkamp and Co., London, UK);
- total carbohydrate content (%) of flours, determined mathematically by subtracting moisture and other quantified macronutrients;
- particle size (Dv90 (µm)) of flours, measured by a laser particle size analyser mod. Mastersizer 3000 (Malvern Panalytical, Malvern, UK) with an obscuration of 0.5-10%, air pressure of 2 bar and hopper height of 3 mm with a feed rate of 50%. The particle sizes were calculated by the supplier's software (version 3.62, Malvern Instruments, Malvern,

UK) and Dv90 (µm), representing the maximum particle diameter below which 90% of the sample falls, was evaluated;

- water holding capacity (WHC) (g of water/g of solid) and water binding capacity (WBC) (g of water/g of solid) of flours, both evaluated in 1 g of flour mixed with 10 mL of distilled water. For WHC the mixture was kept for 24 h at room temperature, then the non-absorbed water was discarded and hydrated sample was weighted. For WBC, the mixture of sample and water was centrifuged for 3 min at 2000 g and 20 °C (mod. Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA), then the non-absorbed water was discarded and hydrated samples were weighted (Sarangapani et al., 2016);
- pH of flours, doughs and biscuits, determined based on the method described by Mesías et al. (2015). Approximately 1 g of grounded sample was mixed with 100 mL of deionized water, vortexed for 3 min and held for 1 h at room temperature. After centrifugation at 4816 rpm and 20 °C for 10 min (mod. Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA), pH of the supernatant was measured with mod. 1100L pH meter (VWR, Radnor, PA, USA);
- colour of raw and baked biscuits, measured using an electronic visual analyser IRIS V400 (Alpha MOS, Toulouse, France) equipped with 25 mm lens and bottom and top lightening. The pre-processing of RGB images and colour quantification in the CIE L*a*b* scale was performed with ImageJ analysis software (NIH, Bethesda, MD, USA). The numerical values of L*, a* and b* parameters were converted into chroma ($C^* = \sqrt{(a^{*2} + b^{*2})}$) and browning index ($BI = ([(X–0.31)\cdot100])/0.17$, where $X = (a^*+1.79\cdot L^*)/(5.645\cdot L^*+a^*–3.012\cdot b^*)$) (Sakin-Yilmazer et al., 2013);
- texture of biscuits, performed at room temperature with Texture analyser TA.XT2 (Stable Micro Systems, Surrey, UK) equipped with a load cell of 50 kg and a three-point bending test holder and probe. The distance of two beams of sample holder was 20 mm and the other setting were: pre-test speed of 5.00 mm/s, test speed of 1.00 mm/s, post-test speed of 10.00 mm/s and distance of 5 mm. The downward movement was advanced till the biscuit was broken. The texture was described by the hardness (N), crispness (linear distance) and fracturability (1/mm) as previously described in section 4.2.2.3 of Chapter 4 (Romani et al., 2012; Tylewicz et al., 2019).

All the analyses were performed in triplicate for each flour and baking batch per sample, except for the determination of macronutrients, colour and texture which were evaluated

in duplicate for each flour, on both surfaces (upper and lower) of 5 biscuits for each baking batch per sample and 8 biscuits for each baking batch per sample, respectively.

5.1.2.3 Asparagine and acrylamide quantification

The sample extraction process for asparagine and AA determinations was optimized according to the method described by Žilić et al. (2020) with small modifications. In detail, 1 g of grounded sample was weighted in 15 mL Greiner tubes and triple extracted with 10 mL, 5 mL and 5 mL of 10 mM formic acid in Milli-Q water. Each time the extract was vortex for 1 min at maximum speed and centrifuged for 10 min at 4816 g and 20 °C (mod. Heraeus Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The combined supernatant was collected in a 50 mL Greiner tube and stored (maximum 2 weeks) in a freezer at −20 °C until analysis.

For asparagine determination, 5 mL of formic acid extract were centrifugated for 10 min at 14000 rpm and 20 °C (mod. 5430 R, Eppendorf AG, Hamburg, Germany). For better clarification, 4 mL of supernatant were centrifugated for 7 min at 14000 rpm and 20 °C, then 1 mL of clear supernatant was mixed with 1 mL of acetonitrile and filtered with 0.2 µm PTFE filters (Ø15 mm) into an amber glass autosampler vial.

For AA quantification, 4.75 mL of the formic acid extract with 100 µL of 5000 µg/L AA-d_3 solution were clarified with 0.125 mL of Carrez I and 0.125 mL of Carrez II. The mixture was vortexed and centrifuged for 3 min at 10000 rpm and 20 °C. For better clarification, 2 mL of supernatant was collected and centrifuged for 10 min at 14000 rpm and 20 °C. For the solid phase extraction clean-up, according to Mogol & Gökmen (2014), the Oasis MCX cartridge (Waters, Milford, MA, USA) was activated with 1 mL of methanol and conditioned with 1 mL of Milli-Q water with a speed of 1 drop/second. Subsequently, 1 mL of clean extract was passed through to preconditioned cartridge (1 drop/second) into an amber glass autosampler vial taking care to discard the first 7-8 drops of the sample to avoid any dilution.

Samples analyses were carried with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, an LC-20ADXR solvent delivery module, a DGU-20ASR degassing unit, a CTO-20AC column oven and an FCV-20AH_2 valve unit.

The chromatographic separation of free asparagine was performed injecting 5 µL of samples on a SeQuant® ZIC HILIC 3.5 µm, 4.6 × 150 mm (Merck KGaS, 64271,

Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 × 2.1 mm (Merck KGaS, 64271, Darmstadt, Germany). The flow rate was set at 0.7 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) with the following elution profile (min/%B): 0.0/90, 4.0/70, 10.0/20, 13.0/20, 15.0/90 and 18.0/90. MS data were collected for 18 min.

The chromatographic separation of AA was performed on Acquity PREMIER BEH C18 column (1.7 µm, 2.1 × 50 mm) connected to an Acquity UPLC BEH C18 VanGuard Pre-column, (130 Å, 1.7 µm, 2.1 mm × 5 mm) (Waters Chromatography B.V, Etten-Leur, The Netherlands) with a flow rate of 0.2 mL/min at 40 °C column temperature. A gradient mixture of mobile phases A (0.1% formic acid) and B (methanol with 0.1% formic acid) was used for elution following the elution profile (min/%B) of: 0.0/5, 2.5/70, 5.0/90, 6.0/90, 7.0/5 and 11.0/5. MS data were collected for 10 min.

Table 5.2 - HPLC-MS/MS acquisition parameters of the optimized SRM transition adopted for the quantification of acrylamide and asparagine.

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Q1 Pre Bias (V)	Q2 (V)	Q3 Pre Bias (V)
Asparagine	133.20	74.00	4	−14.0	−15.0	−13.0
	133.20	87.05	4	−14.0	−12.0	−16.0
	133.20	28.15	4	−10.0	−25.0	−28.0
Acrylamide	72.00	55.10	42	−30.0	−15.0	−23.0
	72.00	27.10	42	−11.0	−23.0	−29.0
	72.00	44.00	42	−12.0	−24.0	−16.0
Acrylamide-d_3	75.25	58.05	42	−30.0	−15.0	−23.0
	75.25	30.05	42	−11.0	−23.0	−29.0
	75.25	44.05	42	−12.0	−24.0	−16.0

Positive ionisation mode was used for the MS analysis. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line and heat block were set at 300 °C, 250 °C and 400 °C, respectively. The pressure of the collision-induced dissociation gas was 4 kPa whereas the flow rates of the drying gas, nebulizer gas and heating gas were set at 10 mL/min, 3 mL/min and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy exit

potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using support software (Shimadzu Corporation, Kyoto, Japan). For single reaction monitoring (SRM), the dwell time was set at 4 or 42 msec, respectively for asparagine and AA, and the most abundant fragment ion was selected for quantitation. The second and third fragments in ion yield were selected as a structural confirmation based on the optimized SRM transition (**Table 5.2**). Data were processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan). The sample extraction was repeated twice for each batch per sample and the analytical measurements were replicated twice for each extract. The results were expressed as µg/kg for AA and mg/kg for asparagine on dry matter basis.

5.1.2.3 Glucose, fructose and sucrose quantification

The sample extraction process for glucose, fructose and sucrose determinations was performed based on Nguyen et al. (2016) with slight modifications. Exactly 2.5 g of grounded biscuit or freeze-dried dough was weighted in 50 mL Greiner tubes, 25 mL of Milli-Q water and ethanol mixture (1:1, v/v) was added and the tubes were vortexed for 1 min. The samples were incubated for 1 h at 50 °C in a water bath and vortexed again before cooled down for 20 min at room temperature. Then the samples were centrifuged at 3000 rpm and 20 °C for 10 min and 1.5 mL of supernatant was collected. Then the supernatant was centrifugated at 14000 rpm and 20 °C for 10 min and 1 mL was collected into a glass tube. The water/ethanol solvent was evaporated with a sample concentrator mod. SBHCONC/1 (Stuart, Staffordshire, UK) under nitrogen flush at 50 °C for 4.5 h. After solvent evaporated, the sample was reconstituted with 20 mL of acetonitrile and 20 mL of Milli-Q water and vortexed for 1 min. Samples were stored in a freezer (−20 °C) until measurement. Before analysis 1.5 mL of sample was passed through CA (Ø28 mm) 0.2 µm filters and transferred into an autosampler vial.

The samples were analysed with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with an Acquity Evaporative Light Scattering (ELSD) detector, an Acquity UPLC BEH Amide column (1.7 µm, 2.1 × 100 mm) and an Acquity UPLC BEH Amide VanGuard pre-column (130Å, 1.7 µm, 2.1 mm × 5 mm) (Waters, Milford, MA, USA). The mobile phase A consisted of Milli-Q water and acetonitrile mixture (8:2, v/v) with 0.2% triethylamine (TEA) while mobile phase B consisted of acetonitrile/water 3:7, v/v with 0.2% TEA. The flow rate was 0.25 mL/min. The gradient changes with the following elution profile (min/%A): 0.00/100, 6.00/40, 6.01/100 and 18/100. Before the first

injection, the column was equilibrated with 100% A, 0.25 mL/min for 30 min. The injection volume was 1.3 µL and the column temperature was 35 °C. Set up a seal wash with acetonitrile/water (1:1), strong needle wash and weak needle wash with acetonitrile/water (8:2) and acetonitrile/water (7:3) respectively. The pressure of ELSD conditions was 40 psi with a drift tube temperature of 40 °C and a data rate 10 pps. Operating the software was carried out using a Waters Acquity Control console and data processing was performed with Chromeleon Chromatography Data System (version 7.2.10, Thermo Scientific Corp, Waltham, MA, USA). The quantification was done by an external calibration curve ranging from 85-1360 mg/L (sucrose) and 45-720 mg/L (glucose and fructose). The sample extraction was repeated twice for each batch per sample and the analytical measurement was conducted twice for each extract. The results for sucrose content of doughs and baked biscuits were expressed as g/kg on dry matter basis.

5.1.2.4 Data analysis

The results were reported as mean value ± standard deviation and were processed as reported in section 3.1.2.5 of Chapter 3.

5.1.3 Results and discussion

5.1.3.1 Flour characterization

The free asparagine content and other characteristics of wheat, lupin and chickpea flours to be related to the AA content and quality characteristics of the final biscuits are presented in **Table 5.3**.

All flours presented protein, fat, fibre and ash contents comparable to those provided by the respective flour manufacturers and other findings in the literature (Cardoso et al., 2019; Hall et al., 2017; Torra et al., 2021; Villarino et al., 2016; Žilić et al., 2020). Wheat and legume flours differed greatly in their protein, lipid, fibre and ash content. Lupin and chickpea flours showed statistically significantly higher values of protein, fat, fibre and ash, while wheat flour showed higher values of carbohydrates. The higher concentration of carbohydrates in wheat flour can be explained by the higher starch content (Hall et al., 2017; Jukanti et al., 2012).

In line with the high protein content of 32.0% and 17.0% of lupin and chickpea flours respectively, the free asparagine content was significantly higher in legume flours than in wheat one.

Table 5.3 - Characterization of wheat, lupin and chickpea flours.

Propriety	Wheat flour	Lupin flour	Chickpea flour
Moisture (%)	12.8 ± 0.1^{a}	6.2 ± 0.1 ^{c}	11.4 ± 0.1^{b}
Water activity (a_w)	0.65 ± 0.0^{a}	0.34 ± 0.01 ^{c}	0.63 ± 0.01^{b}
Protein (%)	11.2 ± 0.0^{c}	32.2 ± 0.3^{a}	17.1 ± 0.2^{b}
Fat (%)	1.2 ± 0.0^{b}	6.1 ± 0.0^{a}	5.9 ± 1.0^{a}
Fiber (%)	2.8 ± 0.8^{c}	40.8 ± 1.2^{a}	11.8 ± 0.7^{b}
Ash (%)	0.6 ± 0.1^{b}	3.0 ± 0.2^{a}	3.1 ± 0.2^{a}
Carbohydrates (%)	71.38 ± 0.66^{a}	11.65 ± 0.90^{c}	50.65 ± 1.06^{b}
Asparagine (mg/kg)	88.4 ± 7.4^{c}	192.2 ± 11.8^{a}	133.7 ± 10.6^{b}
Particle size (Dv90 (µm))	156.3 ± 1.5^{c}	210.3 ± 4.5^{b}	410.0 ± 3.5^{a}
WHC (g water/g solid)	2.0 ± 0.1^{b}	4.7 ± 0.1^{a}	1.8 ± 0.1^{b}
WBC (g water/g solid)	0.7 ± 0.0^{c}	1.7 ± 0.0^{a}	0.9 ± 0.0^{b}
pH	6.1 ± 0.0^{b}	6.0 ± 0.0^{c}	6.7 ± 0.0^{a}
Lightness (L*)	82.1 ± 0.6^{a}	73.3 ± 0.9^{b}	74.8 ± 0.1^{b}
Green-red parameter (a*)	−0.6 ± 0.1^{a}	−1.0 ± 0.2^{b}	−1.2 ± 0.1^{c}
Yellow-blue parameter (b*)	8.8 ± 0.2^{c}	22.9 ± 1.1^{a}	21.5 ± 0.0^{b}

Different letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The amount of free asparagine determined in wheat flour agrees with the results reported by Hamlet et al. (2008), Capuano et al. (2009) and Žilić et al. (2020). Instead, the asparagine levels found in the lupin and chickpea flours of 28.2 mg/kg and 76.5 mg/kg respectively, deviated highly from the values reported by Bartkiene et al. (2016) and Miśkiewicz et al. (2020). However, free asparagine accumulation in crops largely depends on growing conditions as well as their processing methods (Curtis et al., 2018; Miśkiewicz et al., 2012; Žilić et al., 2020). The flours also highly differed in their fibre content from 40.8% in lupin to 2.8% in wheat flour. In general, wheat cereal is naturally a good source of dietary fibre and proteins; however, part of these essential nutrients is substantially lost during milling (Herrera & Gonzalez de Mejia, 2021).

Besides macronutrients, the studied flours differed also in their particle size, the chickpea flour resulted in the highest Dv90 value, indicating that 90% of the sample had a size of 410 µm or smaller. In contrast, wheat flour had the lowest particle size. The moisture and water activity of the flours ranged between 6.2% (lupin flour)-12.8% (wheat flour) and 0.34 (lupin flour)-0.65 (wheat flour). The discrepancies found in the studied flours are probably

due to the different composition, as well as different milling processing conditions. Regarding the hydration proprieties, the lupin flour presented the highest value of WHC and WBC, probably ascribed to the low moisture (6.2 ± 0.1%) and water activity (0.34 ± 0.01) of this flour compared to the others. Wheat and chickpea flours presented very similar WHC and WBC values and differed significantly only for WBC. These hydration properties may also result from the presence of various types of hydrophilic carbohydrates and the varying structure of proteins (Farooq & Boye, 2011). pH values of the water-soluble fraction of the flours were close to neutral, however, the pH of chickpea flour was significantly higher than that of wheat one, while that of lupin was significantly lower. Legume flours were both less light with lower a* and higher b* colour values due to their yellowish colour compared to wheat flour which remains lighter and whiter. The presence of a range of pigments in the cotyledons and seed coats of several legumes gives them a distinct colour. In fact, several seed legumes can be employed not only to improve the nutritional value but also as natural colourants that contribute to the attractive appearance of several food products (Teterycz et al., 2020).

5.1.3.2 Standardisation of asparagine content in the dough

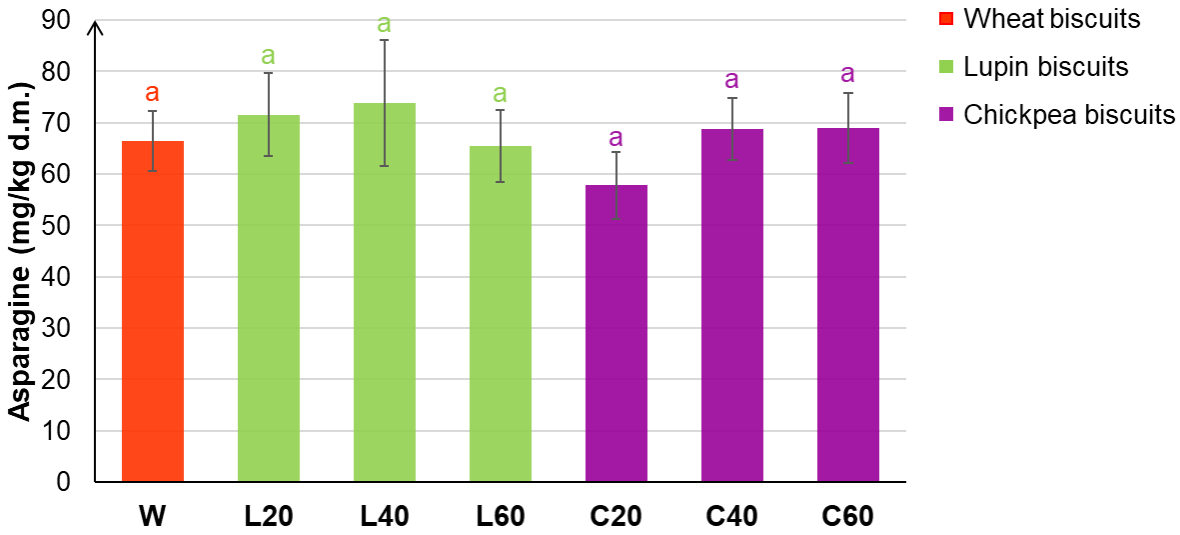


Figure 5.1 - Asparagine content (mg/kg) expressed on dry matter (d.m.) basis in freeze-dried doughs prepared with different flours (W: only wheat; L20: 20% lupin; L40: 40% lupin; L60: 60% lupin; C20: 20% chickpea; C40: 40% chickpea; C60: 60% chickpea). Different letters indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The limiting factor for the formation of AA in biscuits and bakery products is, in general, the content of free asparagine in the flour used (Žilić et al., 2020). However, other flour characteristics can lead to different rates of AA formation (Miśkiewicz et al., 2020). Therefore, to assess the effect of other flours proprieties on AA formation independently from their asparagine content, the quantity of this amino acid in all biscuit doughs was standardized. The amount of free asparagine in each formulation was calculated based on the flour values reported in **Table 5.3**. Consequently, the highest asparagine content was in the L60 dough of about 70.00 mg/kg. Based on this maximum value, the amount of additional free asparagine to be added in the other biscuit types L, C and W were calculated. The asparagine values in the dough samples after standardisation are shown in **Figure 5.1**.

5.1.3.3 Influence of legume flours on acrylamide and precursors contents

The levels of AA, asparagine and sucrose of the biscuit samples during baking at different times are reported in **Table 5.4** and **Table 5.5** for the L and C biscuits, respectively, compared to the control sample W.

Table 5.4 - Acrylamide (µg/kg), asparagine (mg/kg) and sucrose (g/kg) contents on dry matter (d.m.) basis in wheat and lupin biscuit samples (W: only wheat; L20: 20% lupin; L40: 40% lupin; L60: 60% lupin).

Baking time (min)	**W**	**L20**	**L40**	**L60**
Acrylamide (µg/kg d.m.)				
5	420.9 ± 2.8$^{c, C}$	438.7 ± 23.0$^{b, C}$	866.1 ± 39.6$^{b, B}$	1150.9 ± 137.2$^{b, A}$
7	485.7 ± 14.5$^{b, D}$	497.9 ± 36.2$^{ab, D}$	871.0 ± 142.6$^{b, B}$	1261.2 ± 135.7$^{ab, A}$
9	582.3 ± 20.3$^{a, C}$	559.6 ± 42.1$^{a, C}$	1075.6 ± 243.9$^{a, B}$	1443.0 ± 95.8$^{a, A}$
Asparagine (mg/kg d.m.)				
5	72.9 ± 7.8$^{a, A}$	64.7 ± 4.7$^{a, B}$	61.1 ± 12.4$^{a, BC}$	55.4 ± 5.3$^{a, C}$
7	70.1 ± 8.0$^{a, A}$	59.3 ± 8.3$^{ab, B}$	55.5 ± 13.5$^{ab, B}$	53.6 ± 6.4$^{ab, B}$
9	49.7 ± 8.5$^{b, B}$	52.7 ± 5.2$^{b, AB}$	43.8 ± 14.5$^{b, B}$	42.69 ± 2.9$^{b, B}$
Sucrose (g/kg d.m.)				
5	192.8 ± 19.6$^{a, A}$	230.1 ± 9.2$^{a, A}$	205.9 ± 11.8$^{a, A}$	237.6 ± 17.9$^{a, A}$
7	195.1 ± 13.4$^{a, A}$	231.1 ± 6.5$^{a, A}$	206.3 ± 8.1$^{a, A}$	236.2 ± 8.2$^{a, A}$
9	194.1 ± 7.0$^{a, B}$	244.0 ± 3.9$^{a, A}$	214.9 ± 8.8$^{a, A}$	226.7 ± 6.4$^{a, A}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Table 5.5 - Acrylamide (µg/kg), asparagine (mg/kg) and sucrose (g/kg) contents on dry matter (d.m.) basis in wheat and chickpea biscuit samples (W: only wheat; C20: 20% chickpea; C40: 40% chickpea; C60: 60% chickpea).

Baking time (min)	W	C20	C40	C60
Acrylamide (µg/kg d.m.)				
5	420.9 ± 2.8$^{c, C}$	232.9 ± 66.0$^{a, D}$	203.9 ± 35.0$^{a, D}$	756.0 ± 53.4$^{a, B}$
7	485.7 ± 14.5$^{b, D}$	227.7 ± 109.6$^{a, E}$	270.6 ± 36.4$^{a, E}$	702.3 ± 42.9$^{a, C}$
9	582.3 ± 20.3$^{a, C}$	354.4 ± 48.7$^{a, D}$	312.6± 102.2$^{a, D}$	629.6 ± 24.6$^{a, C}$
Asparagine (mg/kg d.m.)				
5	72.9 ± 7.8$^{a, A}$	77.3 ± 2.3$^{a, A}$	67.5 ± 0.9$^{a, AB}$	61.4 ± 0.5$^{a, B}$
7	70.1 ± 8.0$^{a, A}$	70.3 ± 5.8$^{a, A}$	60.9 ± 4.0$^{b, AB}$	56.8 ± 6.3$^{b, AB}$
9	49.7 ± 8.5$^{b, B}$	55.5 ± 1.9$^{b, A}$	58.9 ± 4.0$^{b, A}$	50.4 ± 6.9$^{c, B}$
Sucrose (g/kg d.m.)				
5	192.8 ± 19.6$^{a, A}$	208.8 ± 10.0$^{a, A}$	207.8 ± 19.9$^{a, B}$	236.0 ± 9.8$^{a, A}$
7	195.1 ± 13.4$^{a, A}$	194.9 ± 20.8$^{a, A}$	206.8 ± 11.6$^{a, A}$	241.6 ± 13.9$^{a, A}$
9	194.1 ± 7.0$^{a, B}$	220.1 ± 13.2$^{a, A}$	213.4 ± 30.3$^{a, A}$	239.9 ± 10.3$^{a, A}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The control sample W showed an increase in AA during baking, reaching 582.3 µg/kg after 9 min. AA values in wheat biscuits were higher compared to the ranges reported in previous studies, probably due to the addition of pure asparagine we did to equalize the asparagine concentrations in all formulations (Manolache et al., 2019; Mesías et al., 2016; Sazesh & Goli, 2020; Žilić et al., 2020). Also, for lupin samples (L20, L40, L60) the AA content increased with an increase in baking time. However, at the same initial asparagine content, no significant differences were found between W and L20 samples, whereas L40 and L60 showed higher AA levels proportional to the amount of legume flour used ($r^2 = 0.99$, $r^2 = 1.00$ and $r^2 = 0.99$, respectively for 5, 7 and 9 min of baking). This result could be due to the higher fibre content of lupin flour (40.8%) compared to wheat one (2.8%). The presence of a high percentage of dietary fibre can reduce water activity during baking, thus promoting Maillard reaction and AA formation (Palermo et al., 2012). Similar results were obtained in the studies of Bartkiene et al. (2013, 2016) who described an increase in AA with increased lupin flour in bread and biscuit products. In more detail, the addition of lupin flour determined a maximum increase in AA content of 43.3% in bread and of 78.5% in biscuits (Bartkiene et al., 2013; 2016). In the study on biscuits, the

increase in AA was proportional to the amount of lupin flour added, by 26.6, 93.5, 112.3 and 123.7 µg/kg AA with 0, 4.4, 8.8 and 13.3% lupin flour, respectively (Bartikene et al., 2016). In these studies, the increase in AA was attributed to a higher asparagine content in lupin flour compared to wheat flour.

Low AA levels were obtained in biscuits formulated with chickpea flour when used at 20 and 40%, confirming a possible effect of chickpea proteins previously described by Miśkiewicz et al. (2020). The authors, using a Differential Scanning Calorimetry (DSC) analysis, reported that the melting point of glucose and fructose with 1% of chickpea proteins extract increased, due to a higher ordering of the crystallographic structures of the carbohydrates. This helped to reduce the reaction speed between reducing sugars and asparagine slowing down the formation of AA (Miśkiewicz et al., 2020). In addition, the lower interaction between AA precursors may also have resulted from the coarsest particle size of the chickpea flour. However, no such effect was noticed for lupin biscuit samples despite a larger flour particle size than wheat flour, indicating a greater effect of fibre content by decreasing the aw as previously explained. When wheat flour was substituted with 60% of chickpea flour, an AA increase was detected compared to wheat, C20 and C40 samples at all baking times, probably because at this chickpea flour percentage, the effect of its fiber content (11.8%) on moisture and aw control prevails over the positive effect of chickpea proteins on AA formation described above (Miśkiewicz et al., 2020; Palermo et al., 2012). The percentages of chickpea flour in the biscuits had a non-significant correlation to the amount of AA determined after baking (r2 = 0.71, r2 = 0.82, r2 = 0.64, respectively at 5, 7 and 9 min). Overall, the AA values in chickpea biscuits measured in this research activity are high compared to the result obtained by Miśkiewicz et al. (2012), probably in relation to differences in the biscuit's formulations, as well as in the baking process parameters.

Concerning the asparagine content, for W it was negatively correlated with AA levels (r^2 = −0.91), indicating the participation of this amino acid in the formation of AA. A similar result was observed with L biscuits, with linear correlation coefficients r^2 of 1.00 for L20, 0.91 for L40 and 0.96 for L60. A relatively good negative linear correlation of AA and asparagine contents was also found between samples C20 and C40, but not for the sample C60 in which AA levels did not vary significantly from 5 to 9 min of baking. However, the percentages of asparagine reduction from the dough (time 0) to biscuit baked for 9 min were much lower in samples C than in samples W and L, especially at flour percentages of 20 and 40. For sample W a reduction in asparagine of 24.5% was

measured, for samples L20 and L40 a reduction of 26.4 and 40.6%, respectively, and for samples C20 and C40 only a reduction of 4.1 and 14.3%, respectively. As hypothesised, if chickpea flour is used at 20 and 40%, some of the asparagine failed to react to form AA in the presence of the chickpea protein.

No reducing sugars (i.e. glucose and fructose) could be detected in either the dough or the biscuits. The sucrose contents found in the different dough samples confirmed the standardisation of the recipe between the different formulations. Moreover, the sucrose content did not change significantly during the baking of the biscuits (**Table 5.3** and **Table 5.4**), as a lack of hydrolysis of sucrose during biscuit baking has already been detected by some previous studies (Gökmen et al., 2007; Graf et al., 2006; Nguyen et al., 2016; **Paper IV**).

5.1.3.4 Influence of legume flours on the main characteristics of biscuits

The different flours studied resulted in variations in moisture (%), water activity (a_w), weight loss (%) and pH of the biscuit samples over the different baking times as displayed in **Table 5.6** and **5.7** for L and C biscuits, respectively.

The amount of water added in the dough recipes was standardised and calculated based on the moisture content of each flour to achieve similar moisture content (around 17%) and similar a_w (around 0.80) in all doughs. This because moisture and a_w are parameters that can influence the Maillard reaction and thus AA formation. The moisture content and a_w of all biscuit samples, as expected, decreased with increasing baking time. For all baking times tested the moisture and a_w of the W samples were significantly higher than of the lupin and chickpea biscuits, except for the C60 sample after 5 min of baking in which the values were significantly the same. The low moisture and a_w values of the lupin and chickpea samples could be related to differences in the macronutrient compositions (e. g. fibre, carbohydrates) and particle size of the legume flours (**Table 5.3**). In lupin samples, both moisture and a_w decreased with the increase of the amount of lupin flour, while in chickpea samples these parameters tended to decrease without a clear trend related to the increased amounts of chickpea flour. As reported in previous studies, the weight loss was greatest in the first few minutes of baking (5 min); the formation of a dry surface layer caused a reduction in water vapour flow although the mass transfer continued until the end of baking (9 min) leading to an increase in weight loss percentage (Thorvaldsson & Skjöldebrand, 1998). The weight loss after baking of the lupin biscuits

was significantly greater compared to wheat samples, while for chickpea ones were no significant differences.

Table 5.6 - Moisture (%), water activity (a_w), weight loss (%) and pH values of wheat and lupin biscuits baked at different times (W: only wheat; L20: 20% lupin; L40: 40% lupin; L60: 60% lupin).

Baking time (min)	**W**	**L20**	**L40**	**L60**
Moisture (%)				
0	17.0 ± 0.1$^{a, A}$	16.8 ± 0.4$^{a, A}$	17.1 ± 0.1$^{a, A}$	17.0 ± 0.2$^{a, A}$
5	9.7 ± 0.3$^{b, A}$	9.0 ± 0.1$^{b, B}$	8.7 ± 0.5$^{b, B}$	8.4 ± 0.4$^{b, C}$
7	5.6 ± 1.1$^{c, A}$	5.2 ± 0.1$^{c, B}$	5.0 ± 0.4$^{c, BC}$	4.4 ± 0.2$^{c, C}$
9	3.0 ± 0.2$^{d, A}$	2.5 ± 0.2$^{d, B}$	2.6 ± 0.3$^{d, B}$	2.2 ± 0.2$^{d, C}$
Water activity (a_w)				
0	0.82 ± 0.01$^{a, A}$	0.82 ± 0.00$^{a, A}$	0.81 ± 0.00$^{a, A}$	0.81 ± 0.01$^{a, A}$
5	0.65 ± 0.01$^{b, A}$	0.61 ± 0.00$^{b, B}$	0.59 ± 0.02$^{b, B}$	0.58 ± 0.01$^{b, B}$
7	0.47 ± 0.01$^{c, A}$	0.41 ± 0.01$^{c, B}$	0.39 ± 0.03$^{c, BC}$	0.37 ± 0.01$^{c, C}$
9	0.21 ± 0.01$^{d, A}$	0.18 ± 0.01$^{d, B}$	0.19 ± 0.03$^{d, B}$	0.19 ± 0.00$^{d, B}$
Weight loss (%)				
5	8.1 ± 0.4$^{c, A}$	8.3 ± 0.2$^{c, A}$	8.9 ± 0.6$^{c, A}$	8.9 ± 0.1$^{c, A}$
7	11.4 ± 0.4$^{b, B}$	12.2 ± 0.1$^{b, AB}$	12.7 ± 0.4$^{b, A}$	13.0 ± 0.3$^{b, A}$
9	14.5 ± 0.4$^{a, B}$	14.8 ± 0.3$^{a, B}$	14.9 ± 0.3$^{a, B}$	15.3 ± 0.1$^{a, A}$
pH				
0	8.6 ± 0.1$^{b. A}$	8.4 ± 0.2$^{b, A}$	7.5 ± 2.0$^{b, A}$	7.8 ± 0.0$^{b, A}$
5	9.5 ± 0.0$^{a, A}$	8.6 ± 0.0$^{a, B}$	8.3 ± 0.0$^{a, C}$	7.9 ± 0.0$^{a, D}$
7	9.4 ± 0.0$^{a, A}$	8.3 ± 0.0$^{b, B}$	8.0 ± 0.0$^{a, C}$	7.5 ± 0.0$^{c, D}$
9	8.8 ± 0.0$^{b, A}$	7.8 ± 0.0$^{b, B}$	7.5 ± 0.0$^{b, B}$	7.1 ± 0.0$^{d, C}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Both L and C biscuit doughs had similar pH values compared to the W dough. After baking, the pH was lower in biscuits with higher amounts of L and C flours and lower amounts of additional free asparagine. In W and some C and L samples, the pH increased after the first 5 min of baking, probably due to a concentration of the matrix caused by the water loss.

Table 5.7 - Moisture (%), water activity (a_w), weight loss (%) and pH values of wheat and chickpea biscuits baked at different times (W: only wheat; C20: 20% chickpea; C40: 40% chickpea; C60: 60% chickpea).

Baking time (min)	W	C20	C40	C60
Moisture (%)				
0	17.0 ± 0.1$^{a, A}$	16.5 ± 0.2$^{a, A}$	17.0 ± 0.1$^{a, A}$	17.0 ± 0.1$^{a, A}$
5	9.7 ± 0.3$^{b, A}$	8.8 ± 0.4$^{b, B}$	8.9 ± 0.1$^{b, B}$	9.4 ± 0.2$^{b, AB}$
7	5.6 ± 1.1$^{c, A}$	5.3 ± 0.0$^{c, C}$	5.5 ± 0.0$^{c, B}$	5.3 ± 0.1$^{c, BC}$
9	3.0 ± 0.2$^{d, A}$	2.8 ± 0.1$^{d, B}$	2.8 ± 0.1$^{d, B}$	2.5 ± 0.1$^{d, C}$
Water activity (a_w)				
0	0.82 ± 0.01$^{a, A}$	0.81 ± 0.00$^{a, A}$	0.81 ± 0.01$^{a, A}$	0.81 ± 0.01$^{a, A}$
5	0.65 ± 0.01$^{b, A}$	0.62 ± 0.00$^{b, B}$	0.60 ± 0.00$^{b, B}$	0.62 ± 0.01$^{b, B}$
7	0.47 ± 0.01$^{c, A}$	0.42 ± 0.01$^{c, B}$	0.42 ± 0.01$^{c, B}$	0.42 ± 0.01$^{c, B}$
9	0.21 ± 0.01$^{d, A}$	0.19 ± 0.01$^{d, B}$	0.19 ± 0.00$^{d, B}$	0.20 ± 0.03$^{d, B}$
Weight loss (%)				
5	8.1 ± 0.4$^{c, A}$	8.5 ± 0.4$^{c, A}$	8.7 ± 0.2$^{c, A}$	8.3 ± 0.2$^{c, A}$
7	11.4 ± 0.4$^{b, B}$	12.3 ± 0.2$^{b, A}$	12.2 ± 0.1$^{b, AB}$	12.4 ± 0.3$^{b, A}$
9	14.5 ± 0.4$^{a, A}$	14.7 ± 0.2$^{a, A}$	14.7 ± 0.2$^{a, A}$	14.7 ± 0.7$^{a, A}$
pH				
0	8.6 ± 0.1$^{c, A}$	8.8 ± 0.0$^{b, A}$	8.9 ± 0.0$^{b, A}$	8.6 ± 0.0$^{b, A}$
5	9.5 ± 0.0$^{a, A}$	9.2 ± 0.0$^{a, B}$	9.0 ± 0.0$^{a, C}$	8.7 ± 0.0$^{a, D}$
7	9.4 ± 0.0$^{b, A}$	8.9 ± 0.0$^{b, B}$	8.9 ± 0.0$^{b, C}$	8.6 ± 0.0$^{b, D}$
9	8.8 ± 0.0$^{c, A}$	8.5 ± 0.0$^{c, B}$	8.4 ± 0.2$^{c, B}$	8.2 ± 0.1$^{c, C}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The strongest pH increase was reached in the W biscuits and might be related to the higher amount of added free asparagine. The pH of the dough was close to the pKa of the asparagine, which could result in protonation of the amino acid during baking and lead to a higher pH for the samples with more asparagine added. From 5 to 9 min of baking, the pH subsequently decreased in all samples. The drop in pH may be an indicator of the Maillard reaction and potentially result from acid formation in the development of retro-aldol intermediates (Brands & van Boekel, 2001; Martins et al., 2000). As expected, the different flours studied resulted in variations in the colour and

texture proprieties of the biscuits as shown in **Table 5.8** and **5.9** for L and C biscuits, respectively.

Table 5.8 - Colour (L*, C* and BI of upper surface) and texture (hardness, crispness and fracturability) parameters of wheat and lupin biscuits baked at different times (W: only wheat; L20: 20% lupin; L40: 40% lupin; L60: 60% lupin).

Baking time (min)	W	L20	L40	L60
Lightness (L)*				
0	72.9 ± 0.3$^{a, A}$	66.4 ± 0.0$^{b, B}$	63.5 ± 0.3$^{b, C}$	60.3 ± 0.2$^{c, D}$
5	73.1 ± 0.4$^{a, A}$	70.5 ± 0.5$^{a, B}$	68.0 ± 1.9$^{a, C}$	66.8 ± 0.6$^{a, C}$
7	67.6 ± 1.8$^{b, A}$	68.8 ± 1.0$^{ab, A}$	67.5 ± 1.9$^{a, A}$	66.5 ± 0.7$^{a, A}$
9	61.5 ± 1.9$^{c. A}$	62.6 ± 1.8$^{c, A}$	62.6 ± 2.7$^{b, A}$	61.7 ± 2.0$^{b, A}$
Chroma (C)*				
0	20.8 ± 0.1$^{d, D}$	29.9 ± 0.0$^{a, C}$	32.7 ± 0.4$^{b, B}$	37.5 ± 0.0$^{b, A}$
5	24.5 ± 0.9$^{c, D}$	28.7 ± 0.3$^{a, C}$	34.9 ± 1.0$^{a, B}$	37.8 ± 0.6$^{ab, A}$
7	28.3 ± 0.6$^{b, C}$	28.6 ± 0.7$^{a, C}$	34.7 ± 0.7$^{a, B}$	38.0 ± 0.5$^{a, A}$
9	29.6 ± 0.7$^{a, C}$	28.7 ± 0.3$^{a, C}$	35.2 ± 0.4$^{a, B}$	37.9 ± 3.5$^{a, A}$
Browning index (BI)				
0	37.4 ± 0.2$^{d, C}$	69.3 ± 9.3$^{ab, B}$	92.1 ± 0.3$^{b, A}$	91.8 ± 0.8$^{b, A}$
5	44.0 ± 2.1$^{c, D}$	55.3 ± 1.3$^{c, C}$	73.9 ± 1.5$^{c, B}$	78.7 ± 3.1$^{b, A}$
7	60.0 ± 3.3$^{b, C}$	59.1 ± 2.5$^{b, C}$	73.5 ± 1.5$^{c, B}$	79.4 ± 1.5$^{b, A}$
9	72.5 ± 4.0$^{a, C}$	69.0 ± 3.2$^{a, C}$	78.8 ± 6.5$^{a, B}$	91.2 ± 3.7$^{a, A}$
Hardness (N)				
5	6.5 ± 1.0$^{c, C}$	6.9 ± 1.3$^{c, C}$	9.5 ± 1.5$^{b, B}$	13.0 ± 1.7$^{c, A}$
7	21.7 ± 3.8$^{b, D}$	24.4 ± 3.4$^{b, C}$	27.1 ± 3.4$^{a, B}$	33.6 ± 2.0$^{b, A}$
9	26.5 ± 2.2$^{a, C}$	27.5 ± 2.4$^{a, B}$	27.9 ± 2.7$^{a, B}$	31.2 ± 3.5$^{a, A}$
Fracturability (1/mm)				
5	0.7 ± 0.2$^{c, A}$	0.6 ± 0.1$^{c, B}$	0.6 ± 0.1$^{b, B}$	0.5 ± 0.1$^{c, C}$
7	0.9 ± 0.2$^{b, A}$	0.8 ± 0.2$^{b, A}$	0.8 ± 0.2$^{a, B}$	0.7 ± 0.1$^{b, C}$
9	1.2 ± 0.1$^{a, A}$	0.9 ± 0.2$^{a, B}$	0.9 ± 0.2$^{a, B}$	1.0 ± 0.2$^{a, B}$
Crispness (linear distance)				
5	9.2 ± 1.4$^{c, B}$	8.9 ± 1.8$^{c, B}$	12.5 ± 1.9$^{c, A}$	13.00 ± 2.8$^{c, A}$
7	25.8 ± 3.8$^{b, D}$	27.6 ± 4.0$^{b, C}$	32.3 ± 4.9$^{b, B}$	37.89 ± 3.9$^{b, A}$
9	33.6 ± 4.3$^{a, B}$	34.6 ± 3.2$^{a, B}$	34.6 ± 5.5$^{a, B}$	42.36 ± 13.6$^{a, A}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

Table 5.9 - Colour (L*, C* and BI of upper surface) and texture (hardness, crispness and fracturability) parameters of wheat and chickpea biscuits baked at different times (W: only wheat; C20: 20% chickpea; C40: 40% chickpea; C60: 60% chickpea).

Baking time (min)	W	C20	C40	C60
Lightness (L)*				
0	72.9 ± 0.3$^{a, A}$	70.1 ± 0.5$^{a, B}$	66.6 ± 0.42$^{b, C}$	62.7 ± 0.3$^{b, D}$
5	73.1 ± 0.4$^{a, A}$	72.3 ± 0.5$^{a, AB}$	70.7 ± 0.6$^{a, B}$	68.1 ± 0.3$^{a, C}$
7	67.6 ± 1.8$^{b, A}$	66.3 ± 2.0$^{b, AB}$	65.4 ± 2.0$^{b, BC}$	63.3 ± 2.1$^{b, C}$
9	61.5 ± 1.9$^{c, A}$	59.4 ± 2.1$^{c, AB}$	58.4 ± 2.2$^{c, B}$	56.0 ± 1.9$^{c, C}$
Chroma (C)*				
0	20.8 ± 0.1$^{d, D}$	22.2 ± 0.2$^{b, C}$	33.3 ± 0.6$^{ab, B}$	35.5 ± 0.2$^{a, A}$
5	24.5 ± 0.9$^{c, B}$	23.9 ± 1.0$^{b, B}$	31.3 ± 0.9$^{b, A}$	32.7 ± 0.6$^{b, A}$
7	28.3 ± 0.6$^{b, B}$	27.0 ± 0.6$^{a, B}$	34.2 ± 0.6$^{a, A}$	34.7 ± 0.4$^{a, A}$
9	29.6 ± 0.7$^{a, B}$	27.2 ± 0.3$^{a, B}$	33.7 ± 0.6$^{a, A}$	33.9 ± 0.7$^{ab, A}$
Browning index (BI)				
0	37.4 ± 0.2$^{d, D}$	43.2 ± 0.7$^{c, C}$	72.9 ± 0.1$^{c, B}$	86.3 ± 0.8$^{b, A}$
5	44.0 ± 2.1$^{c, C}$	42.2 ± 1.2$^{c, C}$	62.4 ± 3.0$^{d, B}$	67.8 ± 1.5$^{c, A}$
7	60.0 ± 3.3$^{b, C}$	58.9 ± 3.6$^{b, C}$	80.4 ± 5.5$^{b, B}$	84.0 ± 4.6$^{b, A}$
9	72.5 ± 4.0$^{a, B}$	69.6 ± 3.3$^{a, B}$	92.8 ± 4.6$^{a, A}$	92.7 ± 3.2$^{a, A}$
Hardness (N)				
5	6.5 ± 1.0$^{c, A}$	6.3 ± 1.2$^{c, A}$	5.6 ± 0.8$^{c, B}$	4.1 ± 0.6$^{c, C}$
7	21.7 ± 3.8$^{b, A}$	21.7 ± 2.0$^{b, A}$	21.4 ± 2.3$^{b, A}$	18.9 ± 2.1$^{b, B}$
9	26.5 ± 2.2$^{a, A}$	26.3 ± 3.3$^{a, A}$	24.6 ± 2.8$^{a, AB}$	24.0 ± 2.5$^{a, B}$
Fracturability (1/mm)				
5	0.7 ± 0.2$^{c, A}$	0.7 ± 0.1$^{c, A}$	0.7 ± 0.1$^{c, A}$	0.7 ± 0.0$^{c, A}$
7	0.9 ± 0.2$^{b, B}$	1.00 ± 0.2$^{b, AB}$	1.1 ± 0.1$^{b, A}$	1.1 ± 0.2$^{b, A}$
9	1.2 ± 0.1$^{a, A}$	1.2 ± 0.2$^{a, A}$	1.3 ± 0.2$^{a, A}$	1.3 ± 0.1$^{a, A}$
Crispness (linear distance)				
5	9.2 ± 1.2$^{c, A}$	8.9 ± 1.4$^{c, A}$	8.3 ± 1.0$^{c, A}$	6.8 ± 0.4$^{c, B}$
7	25.8 ± 3.8$^{b, A}$	26.5 ± 3.4$^{b, A}$	26.4 ± 4.0$^{b, A}$	23.0 ± 2.0$^{b, B}$
9	33.6 ± 4.3$^{a, A}$	33.0 ± 5.6$^{a, A}$	33.2 ± 5.7$^{a, A}$	32.3 ± 3.8$^{a, A}$

Different lowercase letters in the same column of each parameter and different uppercase letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The lightness (L*) of the dough and the biscuits decreased significantly with the increasing amount of legume flours used in the formulation and with the increasing of baking time.

Similar results were previously described by Bartkiene et al. (2016) analysing biscuits obtained with lupin flour. However, after 9 min of baking, no differences in L* were noted between the W and L biscuits, whereas significant differences remained with the C biscuits when chickpea flour was used at 40 and 60%. The samples also differed in terms of the parameter C* that increased as the amount of legume flour used increased. In fact, legume flours showed a more yellow and intense colouring than the matt and greyish wheat flour (**Table 5.3**). However, no significant differences in C* were observed between W and sample L20 after 7 and 9 min of baking and sample C20 for all baking times studied. When comparing L and C biscuits, in general, the first samples presented significantly higher L* and C* values than those prepared with chickpea flour. The BI values also followed a similar trend over the percentage of legume flour in the biscuits. For samples formulated with the legume flour (L20, L40, L60 and C40, C60), BI decreased after 5 min of baking compared to the raw biscuit.

This result can be attributed to the lightness of the surface of the biscuits baked at 5 min which was greater than in the raw dough. Probably, the explanation of these L* and BI behaviours are related to physical changes occurring on the surface of the product, such as surface drying and initial volume change (Broyart et al., 1998; Lara et al., 2011; Shibukawa et al., 1989). The subsequent baking stages were characterised by a browning increase of the biscuit surfaces resulting from the Maillard and caramelisation reactions (Lara et al., 2011). The colour of the lower surface of the biscuits was darker and less uniform with a similar colour pattern to the upper surface of the biscuits. The differences in visual appearance of W, L and C biscuits are shown in **Figure 5.2**.

The characteristics of the biscuit texture are reported in **Tables 5.8** and **5.9**, in terms of hardness (N) and fracturability (1/mm) that indicate the firmness of the structure and crispness that is a measure of crumb friability. Generally, hardness is often considered an undesirable characteristic of biscuit products, while fracturability is related to a pleasant sensory characteristic as long as it does not become excessive (Zoulias et al., 2000). For all formulations, biscuits increased in hardness, fracturability and crispness values with increasing baking times as noted in previous research (Lara et al., 2011; **Paper IV**). Through a sequence of physical, chemical and biochemical reactions, the baking process irrevocably changes the structural composition of the biscuits depending on the time-temperature conditions and the formulations used. It is known that oven heat is responsible for the formation of dry surface, volume expansion, coagulation of proteins,

gelatinization of starch and the stabilization of the colloidal dough network (Freeman & Shelton, 1991; Lara et al., 2011).

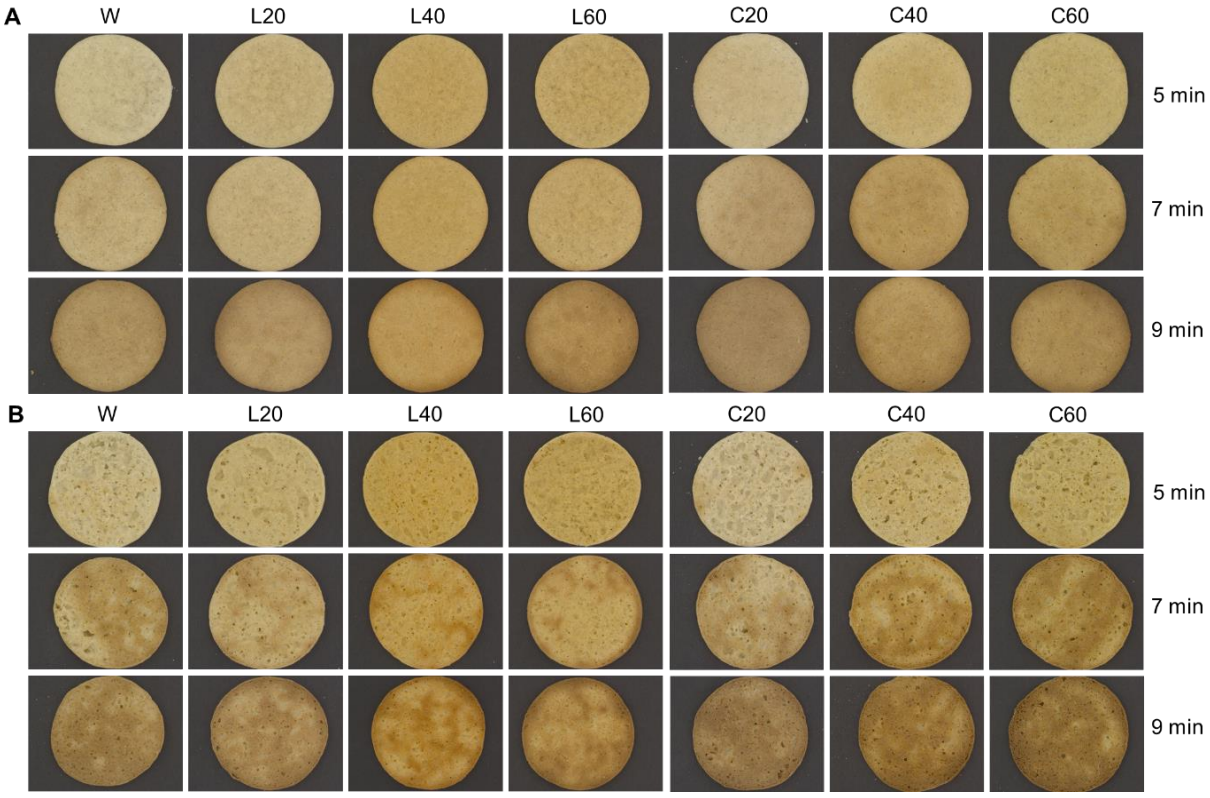


Figure 5.2 - Visual appearance of the upper (A) and lower (B) surface of biscuit samples formulated with different flours (W: only wheat; L20: 20% lupin; L40: 40% lupin; L60: 60% lupin; C20: 20% chickpea; C40: 40% chickpea; C60: 60% chickpea) and baked for different times.

Hardness increased significantly when the amount of lupin flour was increased but decreased with more chickpea flour (60%). As a consequence, the harder biscuits were generally also the less fracturable ones (**Table 5.8** and **Table 5.9**). The highest fibre content, WH and WB capacities identified in lupin flour, as well as the greater chickpea particle size, may have contributed to this texture results. The crispness was significantly different between W and L samples after 5 and 7 min baking times and for lupin flour percentages of 40 and 60%. On the other hand, W samples differed significantly, although slightly, compared to C samples only when chickpea flour was used at 60% for all tested baking times.

5.1.4 Conclusions

The following conclusions can be derived from the findings of this research activity:

- the standardization of the initial asparagine content in the different formulations has been an effective approach to assess the flours effect on AA formation in biscuits;
- lupin flour was not effective in reducing AA in biscuits at all percentages tested. Especially at high concentrations, lupin flour accelerated the AA formation reactions probably due to its higher fibre content causing lower moisture content and aw in biscuits;
- on the other hand, chickpea flour was a promising strategy for controlling AA formation when used at 20 and 40%; in these samples, in fact, were found lower AA concentrations than the reference value reported in Commission Regulation (EU) 2017/2158 (350 µg/kg). The AA reduction using chickpea flour was attributed to the lower interaction between asparagine and sugars resulting from both the coarser particle size and the lower reactivity of carbohydrate in presence of chickpea proteins;
- the use of low portions of chickpea flour did not substantially change the main quality characteristics of the final biscuits. However, further research is also needed to evaluate the effect of the flours used on the sensory properties and consumers' acceptance of the biscuits.

Further studies are needed to evaluate the sensory acceptability of chickpea addition to the biscuits; however, the use of chickpea flour can be a simple and effective solution to mitigate the AA formation in biscuits and other low moisture bakery products.

5.2 Effect of different red kidney bean ingredients in biscuits

5.2.1 Introduction and aim of the research activity

As reported in various studies in the literature, the origin, milling process and pre-treatments (e.g. fermentation) of the flour used in the formulation of some bakery products have a significant influence on the formation of AA. For example, replacing wheat flour with an alternative legume flour, such as chickpea one, can lead to a reduction in AA formation in different food products thanks to some proteins that could bind to carbonyls and thus reduce their availability for the reaction with sugars (Miśkiewicz et al. 2012; 2020; **Paper VI**). Moreover, it has been established that the use of flour with a higher degree of extraction and therefore lower ash content has a higher concentration of asparagine leading to higher levels of AA in the final bakery products (C. L. Fernandes et al., 2019; Mustățea et al., 2016; Negoiță et al., 2016, 2017). The asparagine level in the flour could also be controlled by performing specific fermentation treatments of the

legume and cereal flours to produce safe and high nutritional value biscuits with a reduced AA content (Bartkiene et al., 2016, 2017).

In the case of legume flours, it has been assumed that the structure of the legume cell can be related to the formation of AA in bakery products. Legumes have a cotyledon structure, found to be responsible for some of their health effects as alteration of gastrointestinal motility, rate of starch digestion and absorption (Jenkins et al., 2002). Recent studies have provided evidence that the presence of an intact cell within plant tissues during digestion restricts the access of digestive enzymes and the hydrolysis of intracellular starch in navy beans (Berg et al., 2012) and red kidney beans (Rovalino-Córdova et al., 2018), intracellular lipid digestion in almond (Grundy et al., 2016) and hazelnut (Capuano et al., 2018) as well as protein digestion in soybeans (Zahir et al., 2018). In these studies, an increase in starch, lipid and protein digestions has been observed when the cell wall structure is damaged by mechanical or enzymatic treatments either before or after cooking. The macronutrients in plant foods may be digested to different extents, depending on the degree of particle size reduction, cell rupturing or disruption within the particle and crowded cellular environment (Bhattarai et al., 2016; Mandalari et al., 2018). The cell walls of legumes are composed of a complex network of polysaccharides (e.g. pectin) which are affected by the storage conditions and become less degradable upon cooking (Huisman et al., 2003; McNeil et al., 1984). Consequently, legume cotyledon cells are able to maintain their intact structure when the legumes are cooked as whole cotyledons (Jones & Boulter, 1983; Rovalino-Córdova et al., 2018; Zahir et al., 2018). Following these studies, it was hypothesised that the use of a legume ingredient characterised by a low cell wall integrity, could lead to increased availability of AA precursors during baking, on the contrary, legume ingredient characterised by an intact cell wall could limit it, reducing AA formation.

The purpose of the present research activity was to examine the effect of legume ingredients with intact and broken cell walls on AA formation in biscuits during baking. To test this, biscuit doughs were prepared, according to the standard recipe, by partially replacing wheat flour with red kidney beans as a mash, obtained by boiling and mashing the beans or as a flour, prepared by grinding the beans with a cryo-mill. The integrity or non-integrity of the bean cell walls of obtained flour and mash were analysed by scanning electron microscopy (SEM). Bean ingredients, doughs and baked biscuits were evaluated for AA content and its precursors as well as for the main final quality parameters.

5.2.2 Materials and methods

5.2.2.1 Preparation of bean ingredients

Dry red kidney beans were purchased from the local market (Wageningen, The Netherlands) and stored in the dark at room temperature until use. According to Rovalino-Córdova et al. (2018), with minor modifications, 100 g of beans were soaked for 15 h in 200 mL of ice-chilled distilled water monitoring and standardising the absorption of water by the beans. Subsequently, the beans were de-hulled by manual separation of the seed coat (moisture = 49.4 ± 1.0% and a_w = 0.97 ± 0.01).

The bean ingredients characterised by intact cotyledons cell walls were obtained by boiling de-hulled beans in distilled water (ratio 2:1, w:v) for one hour with gentle magnetic stirring to avoid burning of the material in the bottom of the beaker and at about 100 ± 1 °C. The cooking temperatures were monitored using a digital thermometer equipped with type K thermocouples mod. RS Pro 206-3722 (RS Components, Corby, UK). The cooked beans, without discarding the water (in order not to remove any quantity of asparagine released into the water), were gently crushed with a mortar and pestle to obtain a uniform mash. The bean ingredients characterised by damaged cotyledons cell walls were prepared by grinding 60 g of de-hulled beans into a fine powder using cryogenic mill mod. 6870D (SpexSamplePrep, Metuchen, NJ, USA) with liquid nitrogen and setting 3 steps of 5 min each at 24 back-and-forth cycles per second (cps).

A portion (about 50 g) of boiled and mashed red kidney beans with a thickness of 1.5 cm were heated at 170 °C for 10 min in an electric oven (mod. OV185C, Inventum, Arnhem, The Netherlands) to test the stability of the integrity of the cotyledon cell walls. This bean sample and a part of each bean ingredient were snap-frozen in liquid nitrogen and subsequently freeze-dried for SEM analysis. The sample was attached on SEM sample holders using carbon adhesive tabs (EMS, Washington, WA, USA). The samples were then sputter coated with 12 nm Tungsten (EM SCD 500, Leica, Vienna, Austria) and analysed in an FEI Magellan 400 field emission scanning electron microscope (2kV, 13 pA) (Magellan 400, FEI, Eindhoven, The Netherlands). Size measurements were performed using the xT microscope control software (FEI, Eindhoven, The Netherlands). All red kidney bean ingredients were prepared and analysed with SEM three times.

5.2.2.2 Biscuit sample preparation

The biscuits were formulated partially replacing the refined wheat flour (Windkorenmolen De Vlijt, Wageningen, The Netherlands) with 14% red kidney beans on dry matter basis

as a mash (MB) or as a flour (FB). In detail, the biscuit doughs were prepared with ingredients purchased from local and online markets (Wageningen, The Netherlands) following the standard AACC method 10-54 (AACC, 2000) recipe with small modifications as reported in **Table 5.10**.

Table 5.10 - Recipes of biscuits used in the experiment.

Ingredient	Bean mash biscuits (MB)	Bean flour biscuits (FB)
Wheat flour	172.0 g	172.0 g
Boiled bean	74.4 g	0
Milled bean	0	56.5 g
Fine sucrose	84.0 g	84.0 g
High fructose syrup	3.0 g	3.0 g
Shortening	80.0 g	80.0 g
Non-fat dry milk	2.0 g	2.0 g
Salt	2.5 g	2.5 g
Sodium bicarbonate	2.0 g	2.0 g
Ammonium bicarbonate	1.0 g	1.0 g
Distilled water	1.54 mL	19.5 mL

The amounts of distilled water added in both formulations were standardised according to the different moisture contents of the added bean ingredients, which were 62.4 ± 0.4% and 50.5 ± 0.2% for mash bean and flour bean, respectively. After accurately weighing all the ingredients, the doughs and raw biscuits were prepared with the Thermomix TM5 (Vorwerk, Wuppertal, Germany) and the pasta machine (Marcato, Campodarsego, Italy) following the procedure indicated in section 5.1.2.1. Biscuits were baked in an electrical oven with convection mode (mod. OV185C, Inventum, Arnhem, The Netherlands) at 170 °C for 5, 7, 9 and 11 min. The biscuits were placed on a baking tray in the middle position inside the oven and for each baking cycle, the air temperature inside the oven chamber was monitored every 20 s using a digital thermometer equipped with type K thermocouples (mod. RS Pro 206-3722, RS Components, Corby, UK). After baking, biscuits were removed from the oven, placed on a grid and kept cooling at room temperature for about 1 h. For each formulation and baking time 6 biscuits were prepared. All biscuit formulation and baking time were performed in triplicate; a total of 156 biscuits were prepared.

5.2.2.3 Main quality parameters analysis

The following analytical determinations were carried out according to the procedures indicated in section 5.1.2.2:

- moisture (%) and water activity (a_w) of de-hulled beans, bean ingredients, raw doughs and baked biscuits;
- weight loss (%) during the baking of biscuits;
- pH of bean ingredients, raw doughs and baked biscuits;
- colour (CIE L*a*b*) of raw and baked biscuits;
- texture (hardness, crispness and fracturability) of baked biscuits.

All determinations analyses were performed in triplicate for each de-hulled bean cycle, bean ingredient and baking batch per sample, except for the determination of colour and texture which were evaluated on both surfaces (upper and lower) of 6 biscuits for each baking batch per sample and 6 biscuits for each baking batch per sample, respectively.

5.2.2.4 Asparagine and acrylamide quantification

The dough and biscuit samples were extracted for asparagine and AA determinations according to the method described by **Paper VI** (section 5.1.2.3).

Samples analyses were carried with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, an LC-20ADXR solvent delivery module, a DGU-20ASR degassing unit, a CTO-20AC column oven and an FCV-20AH$_2$ valve unit. For chromatographic separation of free asparagine, the columns and separation conditions indicated in section 5.1.2.3 were used. For AA quantification 5 µL of samples were injected on a ThermoScientific Hypercarb column (5 µm, 2.1 x 50 mm) connected to an OPTI-GUARD® 1mm Guard C18 column. The flow rate was set at 0.2 mL/min and the column temperature at 40°C. The mobile phases consisted of 0.1% formic acid (solvent A), methanol with 0.1% formic acid (solvent B) with the following elution profile (min/%B): 0.0/5, 2.5/5, 5.0/50, 6.0/50, 7.0/5 and 11.0/5. MS data were collected for 10 min. Positive ionisation mode was used for the MS analysis. The voltage of the turbo ion-spray ionization was 4.0 kV. The temperatures of the electrospray ionization probe, desolvation line and heat block were set at 300 °C, 250 °C and 400 °C, respectively. The pressure of the collision-induced dissociation gas was 4 kPa whereas the flow rates of the drying gas, nebulizer gas and heating gas were set at 10 mL/min, 3 mL/min and 10 mL/min, respectively. The electrode

voltage of Q1 pre bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using support software (Shimadzu Corporation, Kyoto, Japan). For single reaction monitoring (SRM), the dwell time was set at 4 or 42 msec, respectively for asparagine and AA, and the most abundant fragment ion was selected for quantitation. The second and third fragments in ion yield were selected as a structural confirmation based on the optimized SRM transition (**Table 5.2**). Data were processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan).
The sample extraction was repeated twice for each batch per sample and the analytical measurement was repeated twice for each extract. The results were expressed as µg/kg for AA and mg/kg for asparagine on dry matter basis.

5.2.2.5 Glucose, fructose and sucrose quantification

The sample extraction process for glucose, fructose and sucrose determinations was performed based on **Paper VI** (section 5.1.2.4). The samples were analysed with an Acquity UPLC-H Class Plus System (Waters, Milford, MA, USA) equipped with an Acquity Evaporative Light Scattering (ELSD) detector with the settings and conditions previously reported in section 5.1.2.3. The sample extraction was repeated twice for each batch per sample and the analytical measurement was done twice for each extract. The results for sucrose content of doughs and baked biscuits were expressed as g/kg on dry matter basis.

5.2.2.6 Data analysis

The results were reported as mean value ± standard deviation and were processed as reported in section 3.1.2.5 of Chapter 3. The relationship between the average values of AA, asparagine, sucrose and some physical characteristics (i.e. weight loss, moisture, a_w and colour parameters) of the biscuits formulated with different bean ingredients and baked under different conditions were evaluated with Pearson correlation coefficient (*r*) with a significance level as reported in section 4.2.2.4 of Chapter 4.

5.2.3 Results and discussion

5.2.3.1 Red kidney bean ingredients characterization

SEM analysis was used to compare the cell wall structure of the bean mash and flour and in **Figures 5.3A** and **B** the related scanning electron micrographs are shown respectively.

SEM images confirmed that the protocols employed were efficient in obtaining intact and damaged cotyledon cell walls of red kidney bean ingredients. In the bean mash (A), the surface of the cells was rough and homogeneous without any crevices while in the bean flour (B) it was not possible to detect the presence of an intact cellular part, the material was found to be completely damaged by milling. In general, legume cell walls are assembled as a continuous network of cellulosexyloglucan in combination with a pectin matrix that serves as filling for the spaces between network constituents, increases cell wall thickness and adds coherence (Cosgrove, 2000; Vincken et al., 2003). During a cooking process, two simultaneous processes occur inside and outside the cotyledon cells; gelatinisation of the intracellular starch and protein denaturation are accompanied by softening as a result of plasticisation or partial solubilisation of the middle lamella, which leads to separation of individual cotyledon cells (Klamczynska et al., 2001; N. Wang, 2008; N. Wang et al., 2003).

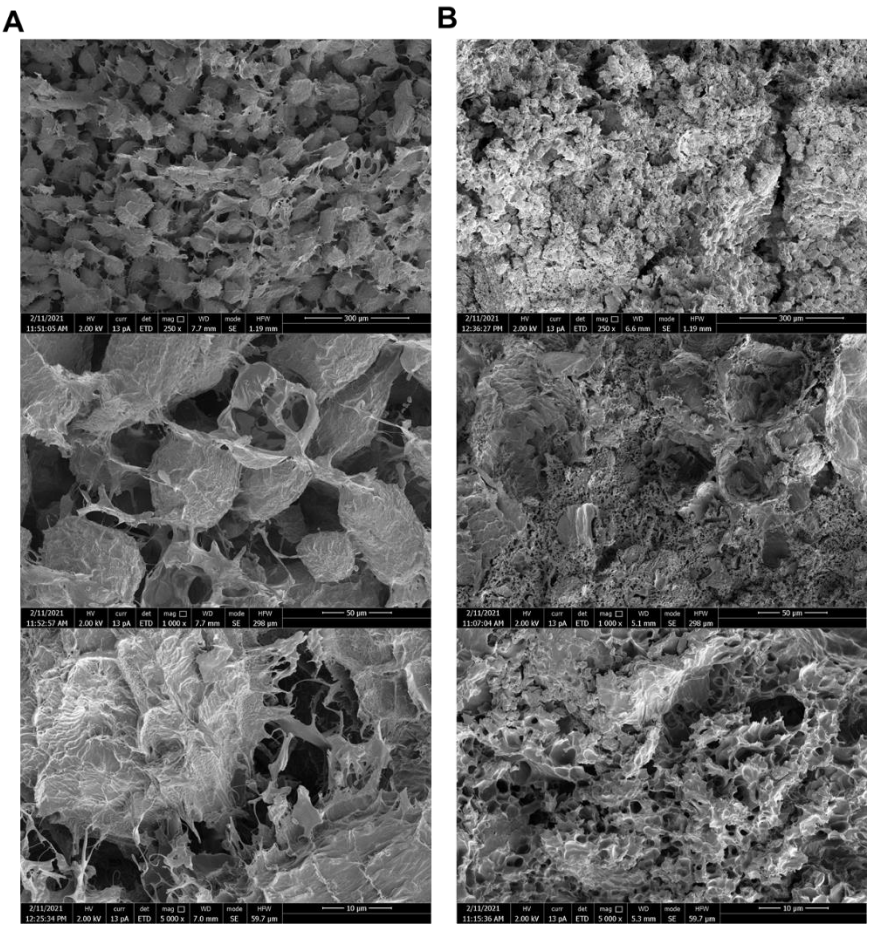


Figure 5.3 - Scanning electron micrographs performed at 250, 1000 and 5000× times magnification of freeze-dried mash (A) and flour (B) from red kidney beans.

However, it has also been confirmed by other studies that low-temperature boiling of cereals and legumes does not lead to a breakdown of the cell walls, but only causes a deterioration of the external appearance and softens the texture (Leelayuthsoontorn & Thipayarat, 2006; Pieniazek & Messina, 2016). The two different preparations here adopted resulted in two ingredients with significantly different moisture contents and water activity values, 63.4 ± 1.1% and 0.96 ± 0.01 for bean mash and 49.6 ± 1.2% and 0.97 ± 0.00 for bean flour, respectively. Moreover, the two bean ingredients also differed slightly but significantly in pH values, 7.0 ± 0.0 and 6.9 ± 0.0 for bean mash and bean flour, respectively. The slightly lower pH of the bean flour can be attributed to the release of cellular contents due to the breakdown of the cotyledons cell walls.

The resistance of the cell structure of the mash beans after heating at 170 °C for 10 min was assessed by SEM. The resulted scanning electron micrographs are reported in **Figure 5.4**.

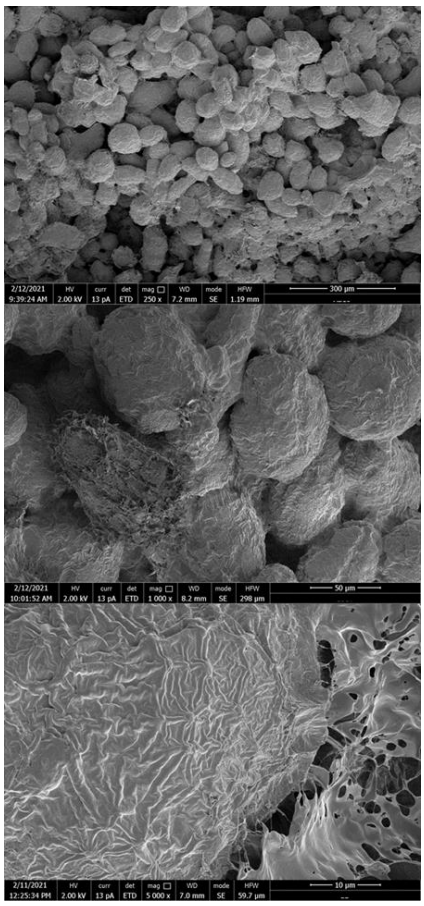


Figure 5.4 - Scanning electron micrographs performed at 250, 1000 and 5000× times magnification of freeze-dried heated mash from red kidney beans.

It was found that the organisation of the cell structure remained unchanged even after heating. In addition, there was no formation of porous cavities on the surface of the cell walls. However, a general superficial difference with the raw mash is appreciable, probably caused by dehydration and loss of water that occurred during the heating treatment.

Following this preliminary study, it was decided to use both the bean ingredients for the formulation of biscuits and to test the two different MB and FB samples for the formation of AA.

5.2.3.2 Influence of red kidney bean ingredients on acrylamide and precursors contents

The behaviours of AA formation in MB and FB biscuit samples during baking at different times are presented in **Figure 5.6**.

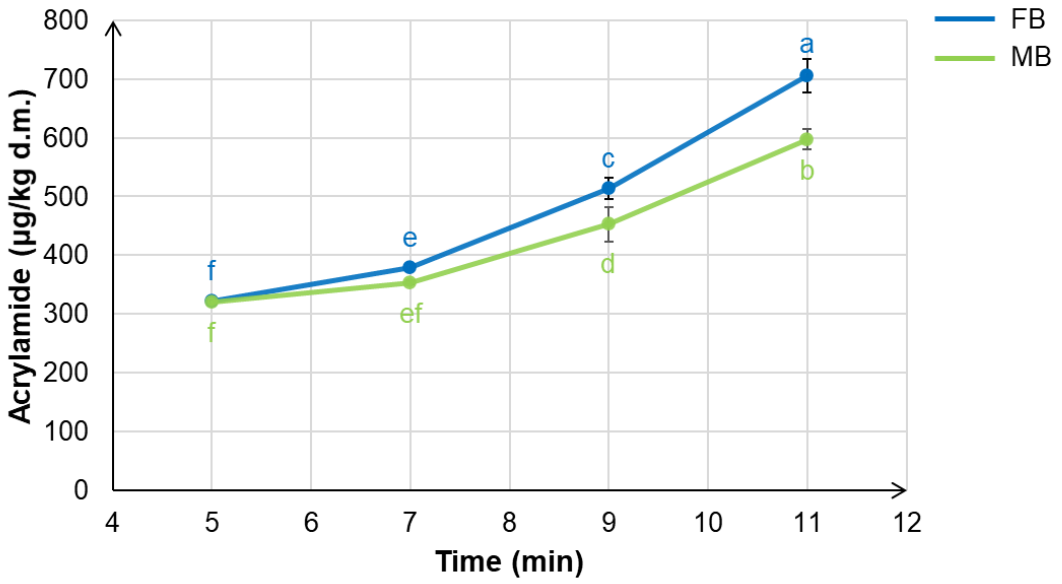


Figure 5.6 - Acrylamide levels (µg/kg) expressed on dry matter (d.m.) basis of bean mash biscuits (MB) and bean flour biscuits (FB) during baking at 170 °C for different times. Different letters indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

As expected, no AA was found in the raw dough samples and there was no significant difference in AA levels between samples MB and FB after 5 and 7 min of baking, with values of 353.9 ± 29.1 µg/kg and 379.3 ± 17.5 µg/kg, respectively. However, at 9 and 11 min baking time, significantly lower AA values were obtained in the sample prepared with the bean mash, although still above the reference values specified in the EU Regulation

2017/2158 for the category “biscuits and wafers” (350 µg/kg). After 9 min of baking, AA levels of 596.8 ± 57.3 µg/kg and 704.9 ± 74.8 µg/kg were achieved for samples MB and FB, respectively. The difference in the rate of AA formation was probably attributed to the higher availability of AA precursors in the milled bean ingredient due to the cell wall breakage. In confirmation of this hypothesis, the levels of asparagine decreased during baking in both formulations, but to a lesser extent for the MB samples as reported in **Table 5.11**.

Table 5.11 - Levels of asparagine (mg/kg) and sucrose (g/kg) expressed on dry matter (d.m.) basis of bean mash (MB) and bean flour (FB) doughs and biscuits during baking at different times.

Baking time (min)	Asparagine (mg/kg d.m.)	Sucrose (g/kg d.m.)
MB		
0	173.9 ± 12.8^{a}	233.6 ± 89.5^{a}
5	178.6 ± 21.5^{a}	213.6 ± 66.7^{a}
7	164.1 ± 17.0^{ab}	205.1 ± 84.0^{a}
9	137.2 ± 15.3^{c}	218.2 ± 69.4^{a}
11	103.7 ± 9.6^{d}	214.9 ± 70.8^{a}
FB		
0	161.1 ± 17.1^{ab}	199.4 ± 50.7^{a}
5	163.2 ± 15.7^{ab}	187.5 ± 24.0^{a}
7	147.9 ± 17.6^{bc}	180.8 ± 27.7^{a}
9	114.8 ± 12.5^{d}	204.2 ± 23.4^{a}
11	81.5 ± 9.2^{e}	187.7 ± 33.8^{a}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

No significant differences between MB and FB in terms of both asparagine and sucrose were determined in the dough (time 0). However, in BM biscuits, asparagine was reduced by 40% and in FB biscuits by 50% after 11 min of baking. No reducing sugars (i.e. glucose and fructose) could be detected in either the dough and the biscuit samples as previously found in **Paper VI**. Probably because of the small amount of reducing sugars in the formulation, these reacted immediately in the first few minutes of baking. Also in this research, the amounts of sucrose measured in the dough were standardised between the two samples. In addition, the sucrose level did not correlate with AA levels ($r = -0.32$) and did not change significantly during the baking of the biscuits, as has been found in

previous studies (Graf et al., 2006; Gökmen et al., 2007; Nguyen et al., 2016; **Paper IV**, **Paper VI**).

5.2.3.3 Influence of red kidney bean ingredients on the main characteristics of biscuits

The evolution of the main quality parameters of biscuit samples, whose data are reported in **Tables 5.12** and **5.13**.

Besides the presence of precursors, the main factor determining the amount of AA formed in biscuits is the presence of water.

Table 5.12 - Moisture (%), water activity (a_w), weight loss (%) and texture proprieties of bean mash biscuits (MB) and bean flour biscuits (FB) baked at 170 °C for different times.

Baking time (min)	Moisture (%)	Water activity (a_w)	Weight loss (%)	Hardness (N)	Crispness (linear distance)	Fracturability (1/mm)
MB						
0	16.4 ± 0.4^{a}	0.82 ± 0.01^{a}	--	--	--	--
5	10.8 ± 0.4^{b}	0.70 ± 0.02^{b}	6.0 ± 0.6^{f}	6.4 ± 1.6^{c}	8.8 ± 1.4^{c}	0.8 ± 0.3^{d}
7	7.2 ± 0.3^{d}	0.54 ± 0.03^{c}	9.6 ± 0.4^{d}	20.3 ± 3.4^{b}	23.2 ± 3.9^{b}	1.1 ± 0.2^{cd}
9	4.5 ± 0.2^{f}	0.35 ± 0.03^{e}	12.2 ± 0.4^{c}	29.9 ± 5.8^{a}	36.1 ± 6.8^{a}	1.2 ± 0.3^{abc}
11	2.5 ± 0.3^{h}	0.19 ± 0.04^{g}	14.3 ± 0.3^{ab}	28.0 ± 3.6^{a}	37.9 ± 9.5^{a}	1.4 ± 0.3^{a}
FB						
0	16.6 ± 0.4^{a}	0.81 ± 0.01^{a}	--	--	--	--
5	9.6 ± 0.2^{c}	0.67 ± 0.01^{b}	7.4 ± 0.5^{e}	5.6 ± 1.2^{c}	8.4 ± 1.6^{c}	0.9 ± 0.3^{d}
7	5.9 ± 0.2^{e}	0.45 ± 0.01^{d}	11.1 ± 0.4^{c}	21.1 ± 4.6^{b}	24.1 ± 4.5^{b}	1.0 ± 0.3^{cd}
9	3.1 ± 0.1^{g}	0.26 ± 0.02^{f}	13.7 ± 0.3^{b}	24.1 ± 2.7^{b}	32.2 ± 6.3^{a}	1.3 ± 0.4^{bcd}
11	1.7 ± 0.1^{i}	0.16 ± 0.01^{g}	15.2 ± 0.4^{a}	22.2 ± 3.0^{b}	31.6 ± 8.0^{a}	1.5 ± 0.6^{ab}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).

The two samples started from similar initial moisture and a_w (about 16 % and 0.80, respectively), both values progressively decreased in both biscuit samples during baking. The moisture content was significantly lower in FB biscuits than in MB biscuits from 5 to 11 min of baking while the aw results became significantly different between the samples only at 7 and 9 min. The faster water loss in the FB biscuits can be probably attributed to cell walls damage of this sample that facilitated water migration and evaporation during biscuit-dough baking. In contrast, in the MB sample, this phenomenon is slower, probably

thanks to the higher water holding capacity exerted by the intact cell walls. The different kinetics of water loss, together with the low availability of asparagine, are the two main factors contributing to the higher levels of AA in the FB sample in agreement with the results of several studies that have reported that low moisture and aw values trigger the Maillard Reaction and thus the formation of AA (Bråthen & Knutsen, 2005; Esposito et al., 2020; Matthäus et al., 2004).

Concerning texture proprieties, the hardness (N), crispness (linear distance) and fracturabilty (1/mm) increased with the prolonging of the baking times in both samples. The biscuits differed significantly only in hardness after 9 and 11 min of baking, when MB biscuits achieved the highest hardness and crispness values despite having higher moisture values than FB ones. This finding can be attributed to the specific structural characteristics of the dough given by the different bean ingredients, making further in-depth studies necessary. On the other hand, there were no significant differences in terms of crispness and fracturability (**Table 5.12**).

Table 5.13 - Lightness (L*), hue angle (h°) and browning index (BI) of upper and lower (**) surfaces of bean mash biscuits (MB) and bean flour biscuits (FB) baked for different times.

Baking time (min)	Lightness (L*)	Hue angle (h°)	Browning index (BI)	Lightness (L*) **	Hue angle (h°) **	Browning index (BI) **
MB						
0	71.7 ± 1.7^{a}	84.7 ± 1.1^{b}	34.2 ± 6.7^{f}	71.7 ± 1.7^{a}	84.7 ± 1.1^{ab}	34.2 ± 6.7^{g}
5	70.5 ± 1.1^{a}	86.4 ± 1.1^{a}	41.5 ± 6.7^{e}	68.1 ± 1.5^{a}	85.8 ± 1.7^{a}	54.3 ± 10.7^{f}
7	66.7 ± 1.2^{b}	83.2 ± 1.1^{b}	55.9 ± 8.7^{d}	61.0 ± 4.8^{b}	82.4 ± 1.5^{b}	71.9 ± 13.4^{e}
9	59.3 ± 1.4^{c}	78.1 ± 0.8^{d}	76.3 ± 8.8^{c}	55.1 ± 5.6^{c}	78.5 ± 1.1^{c}	95.1 ± 24.9^{cd}
11	54.8 ± 1.7^{e}	75.4 ± 0.7^{f}	81.1 ± 15.4^{b}	48.5 ± 5.8^{de}	75.4 ± 1.2^{d}	108.6 ± 25.8^{bc}
FB						
0	69.8 ± 0.9^{a}	83.9 ± 1.0^{b}	33.3 ± 5.8^{f}	69.8 ± 0.9^{a}	83.9 ± 1.0^{ab}	33.3 ± 5.8^{g}
5	71.3 ± 0.6^{a}	86.3 ± 1.0^{a}	44.5 ± 2.7^{e}	68.8 ± 0.5^{a}	86.4 ± 1.3^{a}	52.9 ± 5.7^{f}
7	65.0 ± 2.0^{b}	80.6 ± 1.4^{c}	69.1 ± 7.3^{c}	62.2 ± 2.9^{b}	83.2 ± 3.4^{b}	84.2 ± 23.2^{de}
9	57.0 ± 1.4^{d}	76.4 ± 0.8^{e}	89.3 ± 12.2^{b}	51.4 ± 1.6^{cd}	76.1 ± 1.1^{cd}	120.0 ± 21.9^{ab}
11	50.6 ± 2.1^{f}	74.4 ± 0.9^{f}	109.3 ± 10.8^{a}	44.2 ± 2.7^{e}	73.3 ± 1.1^{d}	138.6 ± 18.2^{a}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p < 0.05$).
** Bottom surface of the biscuits.

As expected, for both samples, the AA content was proportional to the surface colour reached by the biscuits during baking as can be seen in the example pictures shown in **Figure 5.7**. According to the general average values, for both biscuit samples, AA was negatively correlated with L* (r = −0.87 and r = −0.91 for the upper and lower surface respectively) and h° (r = −0.79 for both upper and lower surfaces) values, indicating a concomitant reduction in lightness and change to a redder hue. In addition, together with an increased formation of AA, the biscuits became browner during baking as indicated by an increased BI (r = 0.91 and r = 0.94 for upper and lower surface, respectively) (**Table 5.13**). The upper surface of FB samples, with a higher AA content, showed less bright and darker colour than MB samples at the same cooking times, although not always significantly for the shorter and longer baking times. Significant higher BI levels were also determined in FB samples compared with MB ones after 9 and 11 min of baking (**Table 5.13** and **Figure 5.7B**). The bottom surface of the biscuits was more similar between the samples in terms of L* and h° values, probably due to the fact that the heat transfer from the baking tray was a determining factor in the colour formation.

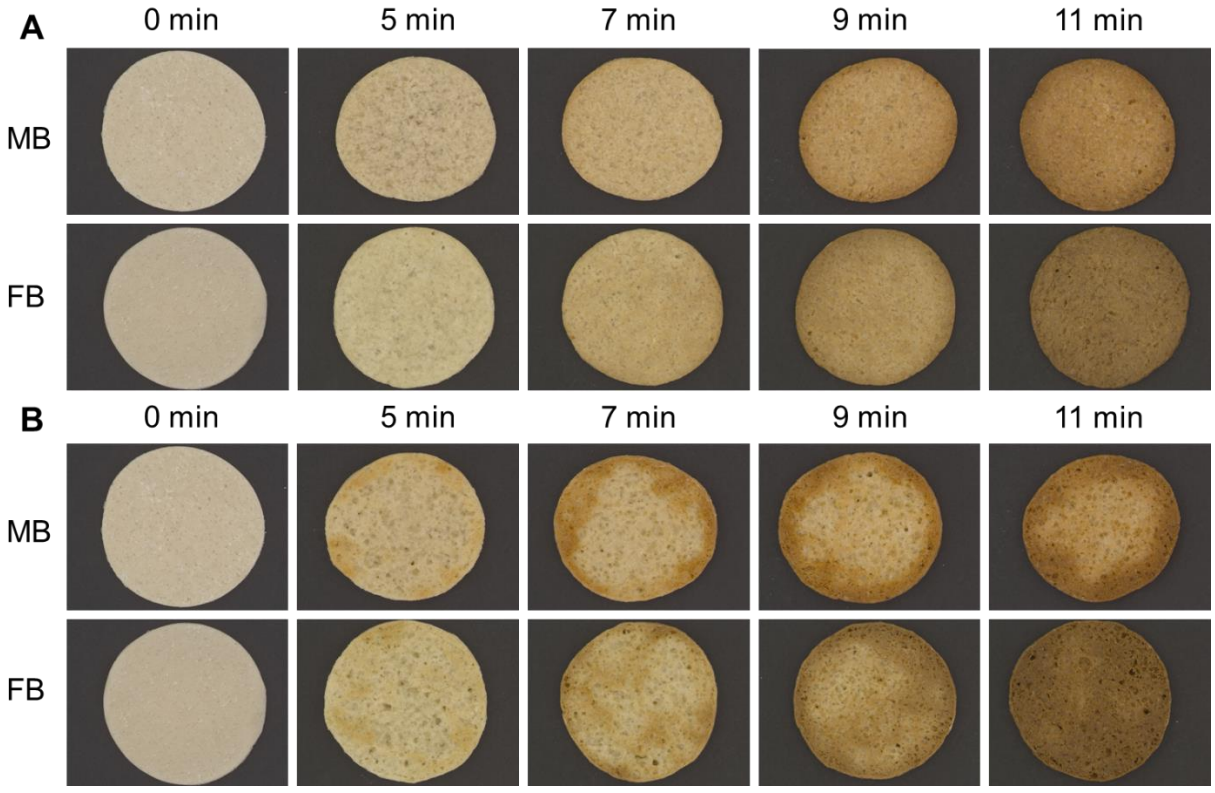


Figure 5.7 - Visual appearance of the upper (A) and lower (B) surface of biscuit samples formulated with 14% of different bean ingredients (MB = bean mash biscuits; FB = bean flour biscuits) and baked at 170 °C for different times.

5.2.4 Conclusions

The following conclusions can be derived from the findings of this research activity:

- the different preparation of the red kidney bean ingredients led to a different cell wall integrity. The results of the SEM analysis confirmed that direct milling of the red beans promoted the mechanical breakdown of the cotyledon cell walls, while boiling the beans before reduction to a mash maintained the integrity of the cell wall;
- the biscuit sample formulated with red kidney bean flours developed a significantly higher level of AA during baking compared to that made with the red kidney bean mash ingredient mainly after 9 and 11 min baking times;
- probably, in intact cells, the precursors of AA are less available during baking and the rate of water loss during baking is slower, reducing the extent of the Maillard reaction;
- moreover, the differently prepared bean ingredients led to different dehydration rates in the biscuit during baking and to different quality characteristics in the cooked products.

The overall results indicated that an accurate design of the legume ingredient preparation (milling and cooking process) could be involved to modulate the AA formation kinetics in biscuit products. In addition, as the two red kidney ingredients also led to different main quality characteristics in the final product, the overall sensory characteristics of biscuits need to be further assessed.

Chapter 6

General conclusions and future perspectives

Since the discovery of the health risks associated with contaminants deriving from conventional cooking processes and in particular high-heat treatments such as frying, baking and roasting of food, there has been a worldwide interest in studying how to prevent them. One of the toxic substances generated by high-temperature cooking processes is AA, classified by the International Agency for Research on Cancer as probably carcinogenic to humans. Nowadays, since food safety remains a primary objective, global food industries, regulatory authorities and institutional communities are increasingly interested in applying interventions aimed at preventing and/or reducing the formation of this compound in order to obtain healthier foods. The main challenge facing the food industry is to minimize these toxicants without adversely affecting desired attributes in cooked foods. So far, several techniques have been proposed for AA mitigation, however, some of these strategies are not easy to apply at the industrial level and sometimes have a negative impact on the organoleptic and nutritional proprieties of the final products. Comprehensive scientific studies are very important to identify and evaluate potential interventions to reduce AA formation in the most at-risk food widely consumed without altering the desired quality characteristics. In this respect, the results of this PhD research project, obtained using innovative pre-treatments and optimising the thermal conditions and formulations, can be summarised in the following relevant outcomes:

- the application of pulsed electric fields (PEF) and yeast immersion pre-treatments, among the recently suggested strategies for AA reduction in potato chips, showed considerable promising results in reducing AA precursors in raw potato tissue by inducing electroporation and through enzymatic activity, respectively. Both the studied pre-treatments did not substantially and negatively influence the main final quality characteristics of the crips such as texture and colour. However, the combination of the two pre-treatments did not show a synergistic effect, leading to lower efficacy in terms of AA reduction compared to the singular treatments. The effect of PEF on yeast activity has to be further studied and elucidated to better exploit the yeast metabolism for AA reduction.
- The selection of roasting degrees represents one of the most promising strategies to reach low levels of AA in roasted coffee products. The AA level and antioxidant activity reached a maximum in coffee roasted at medium degree and then decreased prolonging the roasting process, both in Arabica and Robusta varieties. Nevertheless, the thermal reduction observed was greater for AA compared to the

antioxidant activity, which was only slightly reduced due to the balance between the degradation and the neo-formation of antioxidant compounds.

The control of heat treatment conditions also helps to reduce the formation of AA in sweet and dry bakery products such as biscuits. The baking process in an oven at ventilated mode distributes the heat more evenly causing a faster temperature rise in the product compared to the static mode, resulting in a higher formation of AA. In addition, the baking process with a static mode oven allowed the biscuits to reach the desired quality characteristics as well as a lower level of AA.

- The optimization of recipe formulation is another crucial factor in controlling AA levels in biscuits and other bakery products, as they can have very different formulations. Partial replacement of wheat flour by chickpea flour was an effective strategy to reduce AA in biscuits, probably resulting from the effect of coarser particle size and the effect of legume proteins on the thermodynamic properties of carbohydrate compounds. Moreover, by adding low portions of chickpea flour to the formulation, the main quality characteristics of the final biscuits were not substantially altered. On the other hand, the use of other legume flour such as of lupin origin in various percentages was not as effective in reducing AA in biscuits. Furthermore, it turned out that an accurate preparation of the legume-based ingredients (e.g. by milling and/or boiling process) could influence the AA formation kinetics in biscuits. In fact, biscuits formulated with red kidney bean flour, characterised by damaged cotyledon cell walls, resulted in significantly higher levels of AA formation than those made with red kidney bean mash ingredient characterised by intact cell walls. The AA precursors present in the intact cells may be less available during kneading and baking than those present in the broken ones. Another reason for these results could be that the two different cell types resulted in different rates of dehydration during baking, possibly the damage to the cell wall facilitated the migration of water accelerating the cooking process and AA formation reaction.

The overall results obtained underline the importance of considering the impact of any mitigation strategy on both AA reduction and desired characteristics of the final products by applying a holistic risk-benefit research approach. Indeed, any change in the selection of ingredients and in processing parameters with the intention to reduce AA could also lead to the reduction in the content of beneficial compounds and/or in the overall quality

of the final products. The results obtained during the three years of research activity have allowed a deeper knowledge of the investigated traditional and innovative AA mitigation strategies which can be extremely promising and useful for both the food industry and international authorities. The next challenges in this direction could be to deepen these studies with sensory evaluations and consumer acceptance tests on the final products and to assess the investigated mitigation strategies at industrial scale and on commercial food products.

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