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Cycle XXXV

SURVEY ON THE PRESENCE OF ACRYLAMIDE IN STREET FOOD MARKETED AND PRODUCED IN ITALY AND APPLICATION OF POSSIBLE MITIGATION MEASURES

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ABSTRACT

Acrylamide is an organic, unsaturated compound with a low molecular weight. It forms naturally during cooking at high temperatures of starchy foods rich in reducing sugars and amino acids such as asparagine. The International Agency for Research on Cancer (IARC) has classified acrylamide as a probable human carcinogen belonging to group 2A, based on its carcinogenicity found in rodents. In 2002, Swedish researchers found the presence of acrylamide in several heat-treated carbohydrate-rich foods. This study raised considerable concern as acrylamide is present in food at considerably higher quantities than other known food carcinogens, such as polycyclic aromatic hydrocarbons and ethyl carbamate. Current epidemiological findings on the presence of acrylamide focus more on large-scale industrial products; on the other hand, there is no scientific evidence for typical gastronomic preparations such as the street food. These gastronomic types involve the use of cooking techniques capable of determining a considerable formation of acrylamide. In addition, the heterogeneity of the ingredients used for the preparation of these products leads to have a greater focus on the presence of this substance. The present study has therefore provided the first scientific findings on the presence of acrylamide in street food marketed and produced in Italy through the development and validation of an LC-MS / MS method. The study involved the collection and production of over 450 street food samples from different parts of Italy as well as products of other nationalities commonly consumed in our country. The samples collected and prepared were extracted following several optimization tests with the QuEChERS technique. An LC-MS / MS technique was developed and validated for the analysis of acrylamide, which offers many advantages, reducing analysis costs. Street food samples that exceeded community-recognized benchmark levels were subjected to mitigation tests such as the modification of cooking times and temperatures etc. The results obtained verified contents over the benchmark levels suggested by EU legislation in courgette flowers, Brezel and wraps (piadine). The mitigation measures showed a decrease in acrylamide production during the different types of cooking ($p>0.05$). The results of the mitigation measures proposed in this study will be useful to provide suggestions on the preparation of these products in order to guarantee a product with an ALARA (As Low As Reasonably Achievable) approach which, in the absence of a precise concentration limit, indicates a keep exposure to this contaminant as low as possible. The application of the mitigation measures proposed in this thesis confirmed the extreme importance of time and temperature in the formation of acrylamide, offering products appreciated by industry experts and thus maintaining the excellent organoleptic properties of the product.

SINTESI

L’acrilammide è un composto organico, insaturo a basso peso molecolare. Esso si forma naturalmente durante la cottura ad alte temperature di alimenti amidacei ricchi di zuccheri riducenti ed amminoacidi come l’asparagina. L’International Agency for Research on Cancer, (IARC) ha classificato l'acrilammide come probabile carcinogeno umano, ovvero appartenente al gruppo 2A, in base alla sua cancerogenicità riscontrata nei roditori. Nel 2002, dei ricercatori svedesi hanno riscontrato la presenza di acrilammide in diversi alimenti ricchi di carboidrati trattati termicamente. Questa scoperta ha destato non poche preoccupazioni poiché l'acrilammide è presente negli alimenti in quantità considerevolmente più elevate rispetto ad altri noti agenti cancerogeni alimentari, come gli idrocarburi policiclici aromatici e il carbammato di etile. Gli attuali riscontri epidemiologici sulla presenza di acrilammide si concentrano maggiormente su prodotti industriali di largo consumo; non si hanno evidenze scientifiche invece per preparazioni gastronomiche tipiche come lo street food. Queste tipologie gastronomiche prevedono l’impiego di tecniche di cottura in grado di determinare una considerevole formazione di acrilammide. Inoltre, l’eterogeneità degli ingredienti impiegati per la preparazione di questi prodotti porta ad avere una maggiore attenzione sulla presenza di questa sostanza. Il presente progetto ha quindi fornito dei primi riscontri scientifici sulla presenza di acrilammide nello street food commercializzato in Italia attraverso la messa a punto e validazione di un metodo LC-MS/MS. Lo studio ha previsto la raccolta e produzione di campioni di street food provenienti da diverse parti d’Italia nonché prodotti di altre nazionalità comunemente consumati nel nostro territorio. I campioni raccolti e preparati sono stati estratti a seguito di diverse prove di ottimizzazione con tecnica QuEChERS.

Per l’analisi dell’acrilammide è stata messa a punto e validata una tecnica LC-MS/MS, che offre molti vantaggi, riducendo i costi di analisi. I campioni di cibo di strada che hanno superato i benchmark levels riconosciuti a livello comunitario sono stati sottoposti a prove di mitigazione come la la modifica dei tempi e temperature di cottura etc. I risultati ottenuti hanno verificato dei tenori oltre i benchmark levels per i fiori di zucca, i brezel e le piadine ($p<0.05$). Le misure di mitigazione hanno apportato delle diminuzioni nella produzione di acrilammide durante le diverse tipologie di cottura. I risultati scaturiti dall’applicazione delle misure di mitigazione saranno utili a fornire dei suggerimenti sulla preparazione di questi prodotti al fine di garantire un prodotto avente un approccio ALARA (As Low As Reasonably Achievable) che, in assenza di un preciso riferimento numerico, indica di mantenere l’esposizione a questo contaminante ad un livello quanto più basso possibile. L’applicazione delle misure di mitigazione proposte in questa tesi ha confermato l’estrema importanza dei cambiamenti di tempo e temperatura nella formazione dell’acrilammide, offrendo comunque prodotti apprezzati da esperti del settore e mantenendo quindi le ottime proprietà organolettiche del prodotto.

1. INTRODUCTION

1.1 Acrylamide

Cooking food, domestic or industrial, is an ancient practice that improves the organoleptic properties, the bioavailability of nutrients and microbiological safety.

Cooking techniques such as baking, frying and roasting are indispensable practices in human daily life, but the exposure of food to high temperatures can induce the formation of new molecules, some of which are potentially carcinogenic, including acrylamide [1].

The discovery of acrylamide in food can be attributed to Swedish researchers [2]. Since then, scientific studies have been conducted on the presence, analysis, exposure, formation mechanisms, possible control measures and toxicology of this molecule. In October 1997, during the construction of the Hallandsås railway tunnel in Sweden, there was a sudden death of livestock and fish. In addition, the workers who took part in the construction of the tunnel began to show signs of numbness in their limbs [3].

The death of the animals was due to the drainage water from the construction of the tunnel to the stream. An analysis of the water in Vadbäcken showed very high concentrations of the components of Rhoca Gil, a toxic sealant containing acrylamide and N-methylolacrylamide. It was evident that the reactive monomers leaked out and spread into the environment due to incomplete polymerization. The site was declared a high-risk area, resulting in a ban on the sale of agricultural products from the region and the inability of the population to use water resources. A study conducted by the University of Stockholm measured the presence of acrylamide linked to the hemoglobin of the blood of tunnel workers [4]. During the comparison between workers and a control group, with subjects who had not undergone to occupational exposure, the presence of acrylamide was found in both groups. This ubiquity of results led to the hypothesis that this substance could be found in common contexts such as the diet. This was confirmed on April 24, 2002 with the discovery of acrylamide in foods treated at high temperatures [3]. Acrylamide (C3H5NO) is an organic compound with a low molecular weight (71.08 g/mol), highly soluble in water, which is formed at temperatures normally higher than 120°C and with a low degree of humidity. At room temperature it is a colorless or slightly white, water-soluble and odorless crystalline solid. Acrylamide is an α,β-unsaturated carbonyl compound with electrophilic reactivity. It can therefore react with nucleophilic groups on biological molecules which can contribute to the generation of its toxic effects [5].

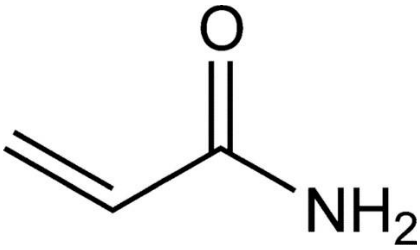


Figure 1. Acrylamide.

Acrylamide is formed by heating foods rich in carbohydrates and asparagine to high temperatures [6]. It is found in heated foods such as chips, french fries, bread, baked goods, breakfast cereals, coffee, chocolate, powdered milk and battered products but also in unheated foods such as prunes and black olives [7].

The sources of exposure are mainly the consumption of a food containing the molecule or the presence of the polyacrylamide binder, an acrylamide polymer, present in the packaging [8]. In the industrial field it is used to form polymers mainly used as coagulants and flocculants in the treatment of drinking and waste water, paper production, waterproofing agents in the textile industry and in the form of gel for electrophoresis in DNA sequencing [7].

The factors that determine the production of acrylamide in the product are amino acids (especially asparagine), reducing sugars and process conditions such as cooking time and temperature, humidity and product composition. It is known that in addition to the presence of native precursors in foods (reducing sugars and asparagine) and/or the process temperature, the low availability of water in foods represents a key factor that triggers the formation of acrylamide. In fact, acrylamide is mainly formed on the external surface of the product where the moisture content is much lower than in the internal part of the food. This lower humidity favors a significant increase in surface area with an exponential generation of acrylamide [9]. It seems that water activity (aw) values lower than 0.4 and higher than 0.8 do not favor the formation of acrylamide [10]. Regarding the pH, the production of acrylamide is maximum at pH values between 7 and 8, while in an acid environment the process is disadvantaged. In addition to being present in heat-treated foods, acrylamide can also be found in unheated foods such as prunes and black olives. In plums and prune juice most likely, the acrylamide comes from sugars and asparagine present in considerable quantities in the raw material [7].

The formation of acrylamide in products subjected to heating is mainly attributable to the Maillard reaction but this is not the only way of formation. The Maillard reaction is a non-enzymatic browning reaction that imparts a unique brownish color and flavor to food products. In addition to having positive effects from an organoleptic point of view, there are also some unwanted consequences such as: loss of nutrients (decrease in the bioavailability of proteins and essential amino acids such as lysine and water-soluble vitamins such as vitamin C) [11], the generation of toxic compounds such as acrylamide and the formation of advanced glycation end products (AGEs) which are responsible for the development of certain age-related diseases.

The Maillard reaction can be divided into three phases: initial, intermediate and final phase [12]. In the initial phase, glycosimine (Schiff's base) is formed by condensation of a reducing sugar, which can be glucose, fructose, maltose or lactose, and an amino group, with the consequent formation of the Amadori compound.

In the intermediate phase there is the development of the intermediate products of the reaction, which give a first light color and a remarkable aroma. Among the main products of this phase are hydroxy-methyl-furfural (HMF) and Strecker's aldehydes and dicarbonyl compounds which are formed from the fragmentation of sugars.

In the final phase there is the condensation of the intermediate products with the formation of melanoidins, which are responsible for the typical colouring.

In a glucose/asparagine system, glucos-1-yl-asparagine undergoes decarboxylation prior to its rearrangement into the Amadori product, to generate N-(D -glucos-1-yl)-3′-aminopropionamide. This intermediate in turn can undergo the Amadori rearrangement to yield N-(1-deoxy-D-fructos-1-yl)-3′-aminopropionamide. Both aminopropionamide intermediates are capable of generating acrylamide either directly or through the formation of free 3-aminopropionamide (Figure 2) [13].

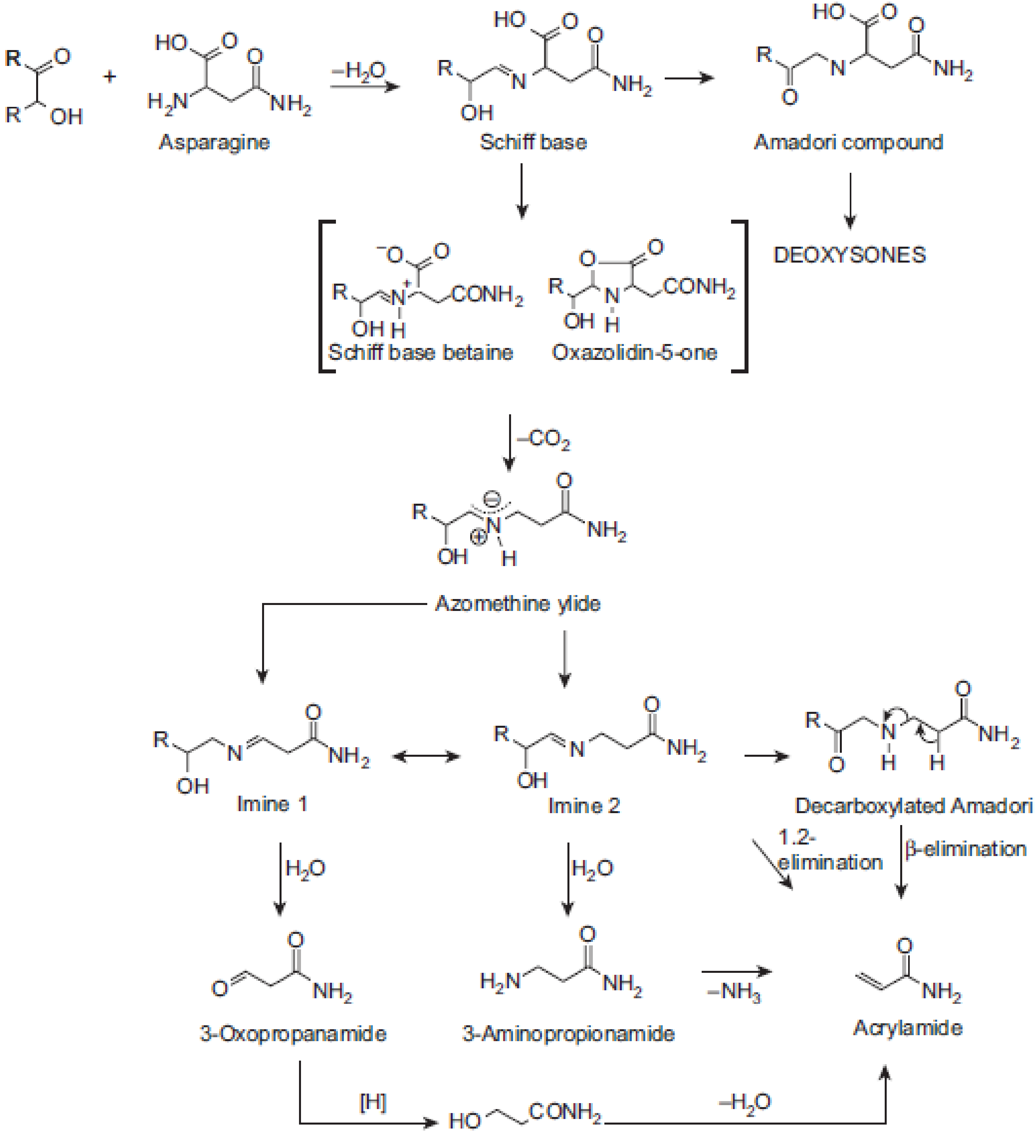


Figure 2. Maillard reaction and acrylamide formation.

The Maillard reaction is not the only formation pathway for acrylamide. In fact, another way of formation is that of lipids which, when subjected to high temperatures, produce large quantities of acrolein. The latter is a volatile aldehyde that at room temperature can be found at liquid form with a characteristic smell of burnt fat. It has a relatively low boiling point (51°C) and its natural source is dehydration of glycerol. It can be formed during the frying of foods, particularly in those cases in which oils or fats with a high content of polyunsaturated fatty acids have been used [14]. Depending on the frying conditions (oil temperature and quality grade), 5 to 250 mg of acrolein per kg of oil can be formed [7]. Frying with peanut oil should guarantee the formation of lower quantities of this aldehyde, which is toxic to the liver and also irritating when inhaled.

Acrolein can produce various intermediates, one of these is acrylic acid which in the presence of nitrogen and heat can lead to the formation of acrylamide.

Acrylic acid can be generated from the oxidation of acrolein, which can further react with ammonia via aminodehydroxylation to give acrylamide. In a typical food matrix, acrolein reacts rapidly with other constituents rather than oxidizing to form acrylic acid. It can also react with asparagine and, under certain conditions, lead to significant amounts of acrylamide.

Acrylic acid requires an additional reaction step, i.e. the conversion of the acid fraction into the corresponding amide, and therefore the availability of ammonia. This is probably the limiting step since ammonia is volatile and not readily available [12]. Thus, lipid systems can also be an indirect source of acrylamide, provided that the acrolein further reacts with amino acids (asparagine) or is oxidized to the intermediate acrylic acid [14].

An additional source of acrylamide besides asparagine and the Maillard pathway are proteins/peptides. Wheat gluten (peptides) can break down under typical cooking or roasting conditions and release acrylamide. A prerequisite for acrylamide release in this pyrolytic pathway is a β-proton in the amino acid adjacent to alanine [15].

The presence of acrylamide in foods was estimated by EFSA (European Food Safety Authority) in 2016 [16]. This estimate shows that fried potato products (French fries, nuggets and baked potatoes) are responsible for up to 49% of the average exposure in adults, followed by coffee (34%), soft bread (23 %) and biscuits, crackers, crispbread and other potato products with a lower incidence.

For children and adolescents, the main source of acrylamide is again represented by fried potato products (except crisps and snacks) up to 51%, followed by soft bread, breakfast cereals, biscuits and other grain or potato products which can contribute up to 25%. In adolescents, the consumption of potato chips and snacks is responsible for up to 11% of the average exposure.

For infants, baby food other than processed cereal-based, potato products and processed cereal-based baby food (especially rusks and biscuits) contribute up to 60%, 48% and 30%, respectively %.

It can be deduced that french fries represent by far the food most at risk for the formation of acrylamide due to the very favorable protein/carbohydrate ratio. It should be noted that although some food categories such as potato chips and snacks and coffee substitutes contain relatively high levels of acrylamide, their overall contribution to dietary exposure is limited if a healthy balanced diet is followed [17].

1.2 Toxicology of acrylamide

The International Agency for Research on Cancer (AIRC) has classified acrylamide among "probable carcinogens for humans" (group 2A), while EFSA, while declaring that the risk is very low, calls for limiting its consumption [20].

According to EC Regulation n. 1272/2008, also known by the acronym CLP (Classification, Labeling and Packaging), acrylamide is classified as a contaminant which:

- H301 toxic if swallowed;
- H312 harmful in contact with skin;
- H315 causes skin irritation;
- H317 may cause an allergic skin reaction;
- H319 causes serious eye irritation;
- H332 harmful if inhaled;
- H340 may cause genetic alterations;
- H350 can cause cancer;
- H361f suspected of damaging fertility;
- H372 causes damage to organs.

Potential routes of human exposure to acrylamide are ingestion, skin contact and inhalation. At the same dose, oral exposure has a greater toxic effect than dermal exposure since the skin can act as a barrier.

The lethal dose (LD50) in rats is 124 mg/kg orally (orally) and 400 mg/kg dermal [5]. Humans can be exposed to acrylamide through diet, drinking water, smoking, secondhand smoke and working conditions. It is also used in some industrial processes such as papermaking, cosmetics manufacturing, drinking water and wastewater treatment [21]. It is found in small quantities in mastic and food packaging materials and some adhesives, as it is used as a building block in the preparation of polyacrylamide and acrylamide copolymers [7].

Once ingested, acrylamide is absorbed from the gastrointestinal tract, distributed to all organs, and extensively metabolised.

It is primarily metabolised through the action of cytochrome P450 2E1 (CYP2E1), which catalyzes the formation of glycidamide (GA), a reactive metabolite of epoxide. Glycidamide binds to DNA and is the most likely cause of gene mutations and tumors seen in animals [22]. Both acrylamide and glycidamide can create adducts with hemoglobin upon reaction with sulfhydryl groups. The abductee level is often used as an indicator of acrylamide exposure since their formation is proportional to the dose of acrylamide ingested, inhaled or absorbed through the skin [23].

In the liver, acrylamide is conjugated with glutathione (GSH), then mercapturic acid is formed to be excreted in the urine [24]. One way to reduce the risk of toxicity could be to increase glutathione levels in the diet by consuming sulfur-containing foods such as onions, garlic, and cruciferous vegetables such as broccoli and Brussels sprouts, or foods that contain significant amounts of the amino acid cysteine, which is an essential substrate for the synthesis of glutathione, such as onions, garlic, cruciferous vegetables and red peppers. Cysteine is also present in foods such as poultry, yogurt and eggs in significant quantities [25]. Precisely for this reason, cysteine is considered a limiting amino acid in the formation of acrylamide.

Acrylamide shows an initial blood half-life of 5 hours and a tissue half-life that can be longer [7].

Regarding the bioavailability of acrylamide, it is 23-44% from food and 32-98% from gavage in rodents [26].

Laboratory animals exposed to oral acrylamide are more likely to develop genetic mutations and tumors (in rats: mammary gland, testicles, and thyroid gland; and in mice: mammary glands, lungs, ovaries, skin, and stomach).

The toxic effect also affects the nervous system (including paralysis of the hind legs), pre- and post-natal development and can negatively affect the male reproductive system.

Although laboratory animal studies have shown that exposure to acrylamide increases the likelihood of developing gene mutations and cancers in various organs, results from human studies provide limited and conflicting evidence regarding an increased risk of developing cancers in association with dietary exposure to acrylamide. However, studies of workers who are occupationally exposed to acrylamide show an increased risk of nervous system disorders manifested by symptoms such as ataxia, skeletal muscle weakness, weight loss, distal swelling and axon degeneration in the central nervous system and peripheral [25].

In animals, acrylamide has neurotoxic effects in mice, rats, guinea pigs, rabbits, cats, dogs and monkeys [27].

From a survey of acrylamide by habitual alcohol consumption, smoking, physical activity, body mass index (BMI), and education to evaluate the average dietary acrylamide intake, it is found that southern and western Europe popluation uptake acrylamide mainly from bread, crispbread, and rusks (24–50%), while northern Europe from coffee (33–40%) followed by bread, toast and rusks (20-35%) [28]. When comparing acrylamide intake with lifestyle factors, alcohol consumption was associated with acrylamide intake in the whole population but more in women who consumed alcohol than in those who abstained. There are insufficient data for men to detect an association between acrylamide intake and alcohol consumption.

As for smoking, smokers are more exposed to acrylamide intake than non-smokers as this substance is found in cigarettes. In fact, blood acrylamide levels in smokers are three to five times higher than those seen in non-smokers [29].

It is almost obvious that physical activity, BMI and education are unrelated to dietary acrylamide intake in both women and men.

Transplacental exposure of fetuses was also evaluated in 2004 to investigate whether free acrylamide in maternal blood was able to cross the human placenta and determine exposure for newborns. If acrylamide is able to cross the placental barrier, it should also be able to form protein adducts and, after metabolic activation to glycidamide, possibly DNA adducts in the fetus.

Specifically, the blood of 11 women in an advanced state of pregnancy (ten non-smokers, one smoker) was analysed a few hours before giving birth, and the corresponding blood from the umbilical cord of 11 newborns. The mean age of the mothers was 31, ranging from 23 to 36 years.

As expected, the highest values for the hemoglobin acrylamide adduct were determined in the blood of the smoking mother and in the corresponding umbilical cord blood of her newborn. Thus, smoking dramatically increases internal exposure to acrylamide for pregnant women, as well as the unborn child. Therefore, due to the increased susceptibility of the fetus to genotoxic substances, transplacental exposure to acrylamide could be of concern [30].

As regards the toxic effects of acrylamide in tissues, different studies have been conducted taking into consideration various tissues. Acrylamide was administered to rats at increasing doses of 0, 5, 15, 30, 45 and 60 mg/kg/day for 5 consecutive days by gavage. Exposure led to morphological changes in testicular histology [31]. In addition, a deterioration in germ cells has been observed following repeated administration of the maximum dose of 60 mg/kg/day.

A large array of multinucleated giant cells with cleaved seminiferous epithelium were observed in the lumen of the seminiferous tubule. In addition, there was a dose-dependent reproductive toxicity affecting the concentration and morphology of sperm present in the cauda epididymis in the treated group [31].

It appears that acrylamide and, therefore, glycidamide are genotoxic and carcinogenic. Consequently, any level of exposure to a genotoxic substance has the potential to damage DNA and cause cancer. However, EFSA scientists concluded that they could not establish a tolerable daily intake (TDI) of acrylamide in food but have estimated the dosage range within which acrylamide is likely to cause a small but measurable incidence of tumors (neoplastic effects) or other potential adverse effects (neurological, pre- and post-natal development and the male reproductive system). The lower limit of this range is called the lower limit of the reference dose (BMDL10) confidence interval. For tumours, the experts chose a BMDL10 of 0.17 mg/kg bw/day. For other effects, the most relevant neurological changes were observed with a BMDL10 of 0.43 mg/kg bw/day [16].

Acrylamide is present in various foods and this makes it clear that this is a transversal problem that cannot be eliminated, but only mitigated through the implementation of guidelines by food industries and food business operators (FBOs).

When we talk about acrylamide reduction, we refer to the acronym “ALARA” (As Low As Reasonably Achievable). This principle states that the FBO should make every reasonable attempt to reduce the levels of acrylamide in the final product, compatibly with what the production process of the food in question allows. Obviously also taking into account the impact on the formation of other contaminants and on microbiological risk, in addition to the company's all-round production needs, to guarantee the health of workers and consumer protection from every point of view, including the nutritional one.

1.3 Legislation

A 2005 EFSA statement revealed that acrylamide is a potential health concern, given its carcinogenicity and genotoxicity in experimental animals. The statement validated the conclusions and recommendations of a previous acrylamide risk assessment study conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In this assessment, JEFCA states that acrylamide may raise concerns for human health and therefore, appropriate measures should be taken to reduce exposure to this substance [32].

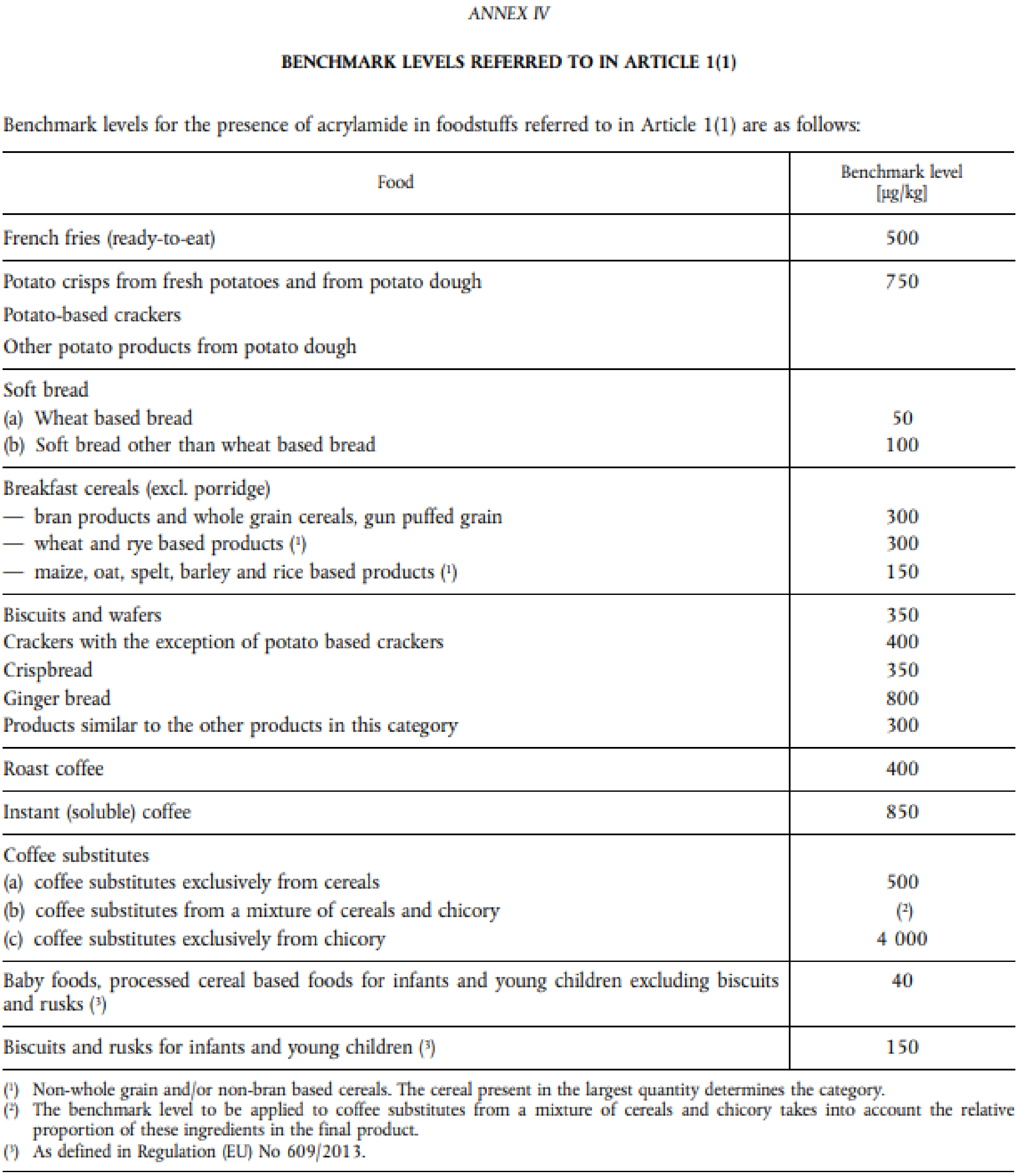
Following a recommendation from the European Commission in 2007, Member States were invited to monitor acrylamide levels over a three-year period and submit the data obtained to EFSA. However, EFSA has no direct role in deciding on such measures.

In 2010, the European Commission recommended that Member States continue monitoring on an annual basis.

Food choice, storage, processing, and cooking method greatly influence the formation of acrylamide and subsequent human exposure. In this regard, Food Drink Europe in close collaboration with national authorities and the European Commission, has developed the Acrylamide Toolbox which helps food manufacturers to identify the best ways to reduce acrylamide in their products taking into account the latest scientific developments and technological. There is talk of reduction and therefore of possible mitigation techniques, as it is not possible to eliminate acrylamide from food. These measures may include, for example, further specific advice or campaigns aimed at consumers on eating and cooking habits at home, or commercial food production controls [33].

In 2015, EFSA published its first comprehensive risk assessment deriving from the presence of acrylamide in food, in which experts concluded that the substance potentially increases the risk of developing cancer in consumers of all age groups [17].

On 20 November 2017, the European Community issued the Regulation (EU) 2017/2158, which establishes measures to reduce the levels of acrylamide in certain food products by setting their reference levels (Table 1). The regulation entered into force on 11 April 2018 [34].



ANNEX IV

BENCHMARK LEVELS REFERRED TO IN ARTICLE 1(1)

Benchmark levels for the presence of acrylamide in foodstuffs referred to in Article 1(1) are as follows:

Food	Benchmark level [µg/kg]
French fries (ready-to-eat)	500
Potato crisps from fresh potatoes and from potato dough Potato-based crackers Other potato products from potato dough	750
Soft bread	
(a) Wheat based bread	50
(b) Soft bread other than wheat based bread	100
Breakfast cereals (excl. porridge)	
— bran products and whole grain cereals, gun puffed grain	300
— wheat and rye based products (1)	300
— maize, oat, spelt, barley and rice based products (1)	150
Biscuits and wafers	350
Crackers with the exception of potato based crackers	400
Crispbread	350
Ginger bread	800
Products similar to the other products in this category	300
Roast coffee	400
Instant (soluble) coffee	850
Coffee substitutes	
(a) coffee substitutes exclusively from cereals	500
(b) coffee substitutes from a mixture of cereals and chicory	(2)
(c) coffee substitutes exclusively from chicory	4 000
Baby foods, processed cereal based foods for infants and young children excluding biscuits and rusks (3)	40
Biscuits and rusks for infants and young children (3)	150

(1) Non-whole grain and/or non-bran based cereals. The cereal present in the largest quantity determines the category.
(2) The benchmark level to be applied to coffee substitutes from a mixture of cereals and chicory takes into account the relative proportion of these ingredients in the final product.
(3) As defined in Regulation (EU) No 609/2013.

Table 1. Benchmark levels proposed by the EU legislation

1.4 Street food

Street food, according to the FAO definition, consists of those foods, including drinks, ready for consumption, which are sold and often also prepared mainly on the street or in other public places such as entertainment areas and festivals, bus terminals, railway stations. Around 2.5 billion people around the world consume street food every day, thanks to its low cost and to meet their nutritional needs away from home. Street food has also become a way to preserve cultural and social heritage and to stimulate tourism. Indeed, it attracts tourists in search of culinary culture and different tastes [18].

The oldest traces of food prepared and cooked on the street date back to ten thousand years ago. The Greeks already described the Egyptian custom, a tradition from the port of Alexandria then adopted throughout Greece, of frying fish and selling it on the street. From Greece the costume passed to the Roman world, enriching itself and transforming itself into countless variations. It is still possible to observe, in the excavations of Herculaneum and Pompeii, the well-preserved remains of typical "thermopolia", the ancestors of the modern "baracchino". These overlooked the street directly, and were used for the sale of cooked foods of all sorts, mainly spelled soups, broad beans or grass peas. Today a particular way of preparing and selling street food with a large vehicle equipped for the preparation and sale of food, which takes the name of "food truck", is spreading more and more. One can thus use the same place every day and recently offers gourmet cuisine and a variety of specialty and ethnic menus.

Street food is considered a food safety risk since vendors often lack basic food service infrastructure and equipment such as storage facilities, food heating equipment and refrigerators [19]. This is also due to the fact that street food vendors often lack training in adequate food safety knowledge, leading to unintentional contamination.

Furthermore, due to their composition (rich in starches and reducing sugars) and their preparation (frying, baking and pan-cooking) these products are subjected to the formation of acrylamide. Among these, french fries are the most famous product that report high quantities of acrylamide.

1.4 Analytical techniques approved for acrylamide detection

According to EU Recommendations, Member States should carry out the analysis of acrylamide according to the criteria set out in points 1 and 2 of Annex III of the EC Regulation 882/2004 on official controls, to ensure the verification of compliance with legislation on feed and food, animal health and animal welfare. This implies that analytical methods should be characterized by at least the following criteria: accuracy, applicability (matrix and concentration range), limit of detection (LOD), limit of quantification (LOQ), precision, repeatability, reproducibility, recovery, selectivity, sensitivity, linearity and measurement uncertainty [35].

The analytical determination of acrylamide in foodstuffs is mainly performed by liquid chromatography (LC) or gas chromatography (GC) methods in combination with mass spectrometry (MS).

1.4.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a technique for separating analytes in a column by interacting with both the stationary (column) and mobile (eluent) phases. The stationary phase retains the components of the sample in different ways, allowing them to be separated.

The solid support of the stationary phase contained in the columns consists of particles of silica or synthetic polymers (cross-linked polystyrene), which can be irregular or spherical in shape and with different diameters. Spherical particles are mostly used, as they have higher packing efficiency and better reproducibility.

Commercially available porous particle columns with pores ranging from 60 to 150 Ǻ for traditional analytical purposes; from 300 to 500 Ǻ for analysis of small peptides and proteins and from 1000 to 5000 Ǻ for macromolecules [36].

The mobile phases used in HPLC generally consist of a mixture of one or more solvents having high purity, low cost, UV transparency, non-corrosive, low viscosity, low toxicity, non-flammable. The strength must be related to the polarity of the solvent (e.g. water is a strong solvent in normal phase and weak in reverse phase). Selectivity depends on interactions such as dipole moment, induced dipole, hydrogen bond.

To modify the mobile phases, it can be used:

- buffers (phosphate, acetate, citrate to stabilize the pH of the reversed phase mobile phase and ion chromatography);
- acidifiers (phosphoric acid, acetic acid to suppress the ionization of acid analytes in reverse phase);
- ion pairing reagents (sulfurized hexane to separate ionic compounds in reverse phase);
- basic modifiers (trimethylamines to suppress the ionization of basic analytes in reverse phase).

As for the stationary phase, silica is characterized by good mechanical strength, reproducibility and high efficiency but has limited pH resistance and some chemical reactivity. Thanks to the extraordinary chemical reactivity of its surface, it is the most used material.

The strengths of polymeric resins are the extended pH range and chemical inertness, while the weaknesses are lower efficiency, lower mechanical stiffness and reproducibility. The activity of a stationary phase is a function of the different functional groups present on the surface of the particles which operate with different separation mechanisms. Analytes, when they come into contact with the stationary phase, can interact in different ways depending on their structure. Nonpolar interactions can occur if the stationary phase is nonpolar; polar interactions if the stationary phase is polar; ionic interactions of the cationic type if the pH is two units lower than the pK and of the anionic type if the pH is two units higher than the pK.

The separation mechanisms can be different (adsorption, normal or reverse phase partitioning, ion exchange, molecular exclusion and immunoaffinity). Depending on the column, one or more mechanisms can be present at the same time.

For the determination of acrylamide, partition chromatography (or liquid-liquid, CLL) was used, based on the different partitioning of the analytes between the stationary and mobile phases, by means of separation mechanisms involving different types of interactions such as the forces of Van der Waals and hydrogen bonding.

There are two types of partition chromatography: normal-phase and reversed-phase.

In normal phase chromatography there is a partition of the analytes between a highly polar solid stationary phase (e.g. silica, alumina, amino, diol) and a predominantly apolar liquid (eluent mixture).

In the reverse phase, a liquid/liquid partition takes place between the non-polar liquid state (e.g. C8, C18) bound to the silica and the polar immiscible mobile phase. The most used mobile phases in this type of separation are aqueous solutions containing solvents such as methanol, acetonitrile or tetrahydrofuran in various concentrations.

In reverse chromatography the most polar component is the one that elutes first and an increase in the polarity of the mobile phase increases the retention time. In the polar phase, the least polar component of the mixture to be separated is eluted first and increasing the polarity of the mobile phase decreases the elution time.

Generally, the equipment of an HPLC consists of a pump system, an injector block, a column, a detector and a data processor (figure 3).

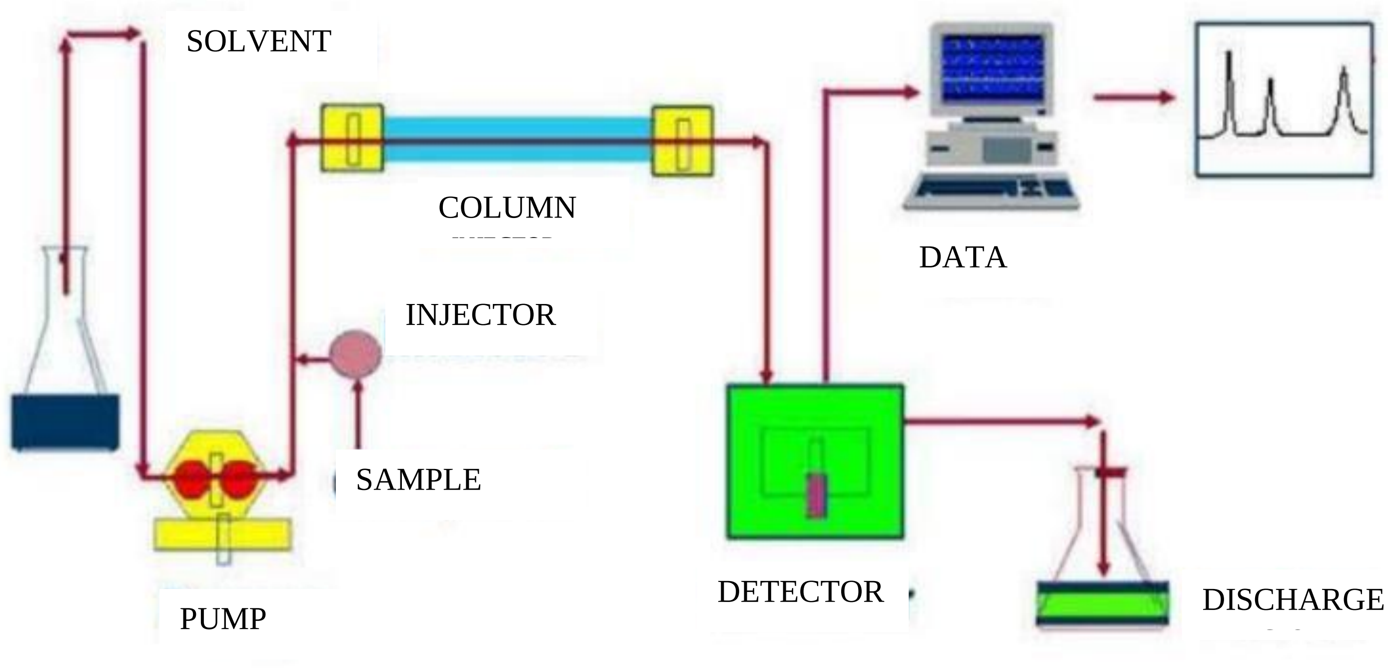


Figure 3. Scheme of a HPLC system.

A modern system is equipped with one or more glass containers, each of which can hold 500 mL or more of solvent. Means are also often provided to remove dissolved gases and dust from the solvents, as the gases can bubble in the column and cause band broadening. Furthermore, both gas bubbles and dust interfere with the detector performance [37]. There are also devices for degassing the eluents by bubbling inert gas (generally helium) inside the container which carries away the dissolved gases or systems which, with a vacuum pump, remove the gases through semi-porous membranes.

An elution carried out using a single solvent or a solvent mixture of constant composition as the mobile phase is called isocratic. In contrast, two (or even more) solvent systems are used in gradient elution, the polarity of which differs significantly. The latter can improve the separation efficiency.

Furthermore, proportional valves allow the introduction of solvents from two or more containers with ratios that can be varied continuously.

There are 4 types of pumps: pneumatic, syringe, hydraulic amplification and reciprocating. Most of the pumps used are reciprocating, which consist of a small cylindrical chamber which is filled and emptied by the reciprocating movement of a piston. Basically, they send it to the column. Pumping produces a pulsed flow which must subsequently be linearised.

There may be two to four pumps in an instrument and the wettable parts must be of inert material, such as stainless steel, Teflon, PEEK (Polyetheretherketone).

The advantages of reciprocating pumps are small internal volume, high external pressures, easy adaptability to gradient elutions, and constant flow rates largely independent of column back pressure and solvent viscosity.

Some instruments use an air operated pump which in its simplest form consists of a compressible container of solvent housed in a vessel which can be pressurized by a compressed gas. Pumps of this type are simple, inexpensive and do not give impulses but have the disadvantages of limited output capacity and pressure and the dependence of the pumping speed on the viscosity of the solvent. Also, they are not suitable for gradient elution.

The injector allows the sample to be introduced directly into the column without interrupting or altering the flow of the eluent mixture.

By far the most widely used method for sample introduction is based on the use of a sampler (loop). Often, interchangeable samplers are available that allow sample sizes from 5 to 500 µL. The reproducibility of injections with a sampler of this type is within a few tenths of a percent. Many HPLC instruments contain an autosampler with an automatic injection system capable of continuously injecting varying volumes. The autosamplers are equipped with holders for 8-100 vials. Regardless of automatic or manual injection, the interface universally used to introduce the sample is the 6-way valve (figure 4), called Rheodyne.

Figure 4. 6 ways valve (Rheodyne).

The column (figure 5) is usually made of stainless-steel tubing but glass or Tygon tubing can also be used. The column is connected on one side to the pump, where there is the inlet of the eluent mixture and on the other side to the detector, where there is the outlet of the eluent mixture with the separate analytes.

At the ends of the column there are porous filters (frit): the head one prevents any solids present in the sample from entering the column and the bottom one prevents the filling from being transported into the detector.

Most of the columns vary in length from 10 to 30 cm and internal diameters from 2 to 5 mm. In general, however, the length of the column varies according to the granulometry of the filling.

A guard column is often introduced at the top of the analytical column, to increase the life of the analytical column through a preliminary removal of particulate matter and impurities contained in the solvents. This also serves to saturate the mobile phase with the stationary phase, thereby minimizing the possibility of stationary phase losses from the analytical column. The packing composition of the guard column should be similar to that of the analytical column, while the particle size is generally larger to minimize the pressure drop.

For many applications tight control of the column temperature is not required and in these cases the columns operate at room temperature. However, better chromatograms are obtained by keeping the temperature constant to within a few tenths of a degree Celsius, as retention times are thus much more reproducible. Modern instruments are equipped with column heaters which can maintain a temperature in a variable range from a few tens of degrees to 150°C but usually working at room temperatures (20-40°C).

Columns can also be equipped with water jackets, warmed by a constant temperature bath, to provide precise temperature control [38].

Figure 5. Column used for acrylamide analysis.

HPLC detectors must have a small dead volume to minimize extra-column band-broadening. The detector should be small and compatible with liquid flow. The choice of detector depends on the nature of the analytes to be identified and their concentration in the analysed food matrices.

Precisely due to the nature of acrylamide and the presence of various interferents within the food matrix, a very selective detector is required which allows the analyte to be precisely qualified and quantified. This detector is the mass spectrometer. The coupling of mass spectrometry to HPLC allows to analyse complex mixtures and identify with absolute precision analytes present even in traces [39].

1.4.2 Mass spectrometry

Mass spectrometry is an analytical technique in which the molecules of a sample are converted into gaseous ions which are then separated according to their mass-to-charge ratio (m/z) [40].

The mass spectrometer is capable of analysing molecules that have one or more positive or negative charges.

The essential features of all mass spectrometers are:

- production of ions in the gaseous phase (most problematic phase);
- acceleration of ions in an electric field;
- ion separation in a mass analyser;
- detection of each species based on the m/z ratio.

The samples must be ionized and converted into gaseous form, after which they enter the mass spectrometer, where the ions are accelerated and separated as a function of their m/z ratio by an analyser. To minimize collisions between ions and air molecules, all mass analysers operate in high vacuum conditions to allow the ions formed in the source to reach the detector with good yield [41].

In order to function properly, the mass spectrometer must consist of a high vacuum system, an introduction system (direct, HPLC or GC), a source capable of producing ions, an optional selective ion fragmentation system, the analyser which separates the generated ions on the basis of the m/z ratio, an ion detection system, a software system capable of recording and processing the very high number of information coming from the detector (figure 6).

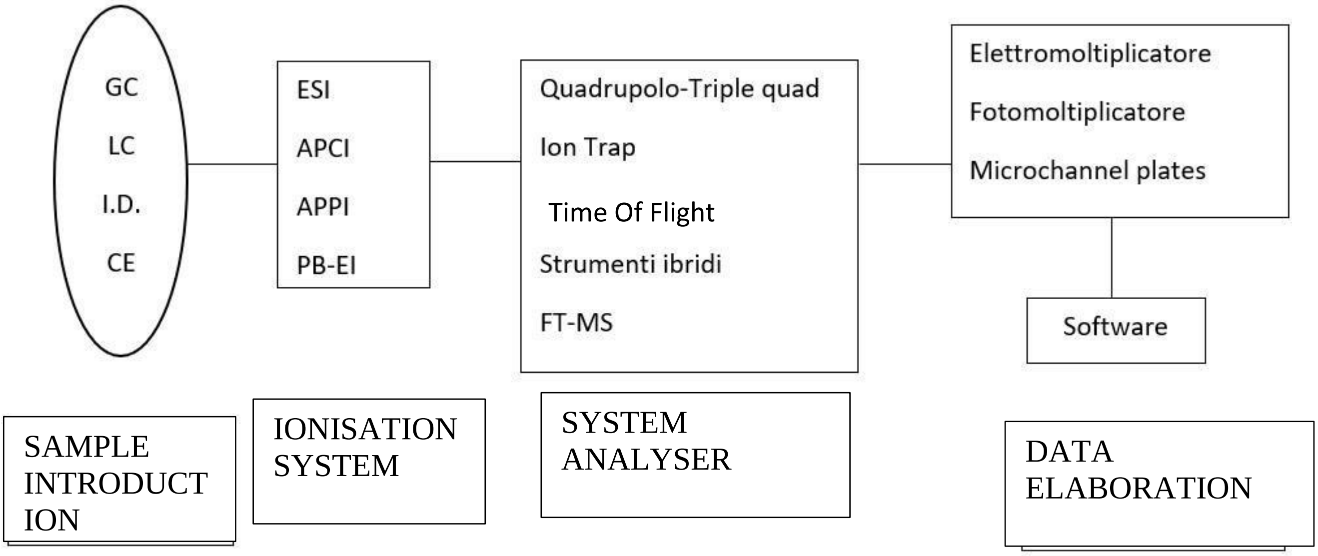


Figure 6. General structure of a massa spectrometer.

HPLC is usually used coupled with detectors capable of providing only little general information about the compounds under investigation.

HPLC-MS is the result of combining the chromatographic capabilities of the resolution of complex mixtures of HPLC with the ability of structural elucidation of mass spectrometry.

The eluate leaving the column and the mass spectrometer work at different pressures.

It is therefore understandable that it is not possible to introduce the eluate from the column directly into the source of the mass spectrometer. The problem was solved in the late 1980s, with the development of ionization by electrospray (ESI).

The first phase represents the production of ions in the gaseous phase; therefore, there must be an LC-MS interface system capable of evaporating the mobile phase and ionizing the analyte. Different ionization methods are available on the market, each of which has strengths and weaknesses.

The most used ionization techniques are:

- electrospray ionization (ESI);
- atmospheric pressure chemical ionization (APCI);
- atmospheric pressure photoionization (APPI);
- matrix assisted laser desorption ionisation (MALDI).

The electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces are the most commonly used in food control laboratories.

The ionization method to use depends on the type of sample and the spectrometer available.

The technique used for the analysis of acrylamide is ESI.

ESI is a technique that produces gaseous positive or negative molecular ions from organic compounds with molecular mass up to 150 KDa (50 Da-150 KDa).

An ESI type ionizer is formed by a capillary that ends in a narrowing which takes the name of Taylor cone, which favors the formation of gaseous particles. The sample is dissolved in a polar and volatile solvent and pumped through a fine stainless-steel capillary (75-150 µm) at a flow rate between 1 and 1000 µL/min. A high voltage (2/5 kV) is applied to the tip of the capillary. As a consequence of the strong electric field, the sample is ionized and dispersed in an aerosol of charged droplets which are directed towards the vacuum chamber, where extremely high temperatures are present (300°C) and this causes the solvent, still present in the droplets, evaporates.

At the exit of the vacuum chamber there is a gaseous spray of tiny droplets comprising only molecules of the sample. The charged droplets decrease in size by evaporation of the solvent in an inert gas, to facilitate evaporation and under vacuum, to prevent collisions between molecules (figure 7).

ESI ionization is coupled with ion trap or quadrupole analysers.

Among the advantages of ESI there is good functioning with ionic or polar analytes, it provides information on the molecular weight, there is little fragmentation and therefore most of the molecules leave the ionizer intact, it is characterized by high sensitivity. Precisely for this reason this type of ionization is used for acrylamide.

However, it is not compatible with the use of apolar organic solvents, it tolerates methanol, acetonitrile but does not tolerate other more apolar solvents such as acetone and hexane. Ionization is inhibited by high salt concentrations, in fact many times the sample is first desalted by dialysis or simply by chromatography. Another disadvantage is that it produces multicharged ions, making subsequent data analysis more difficult.

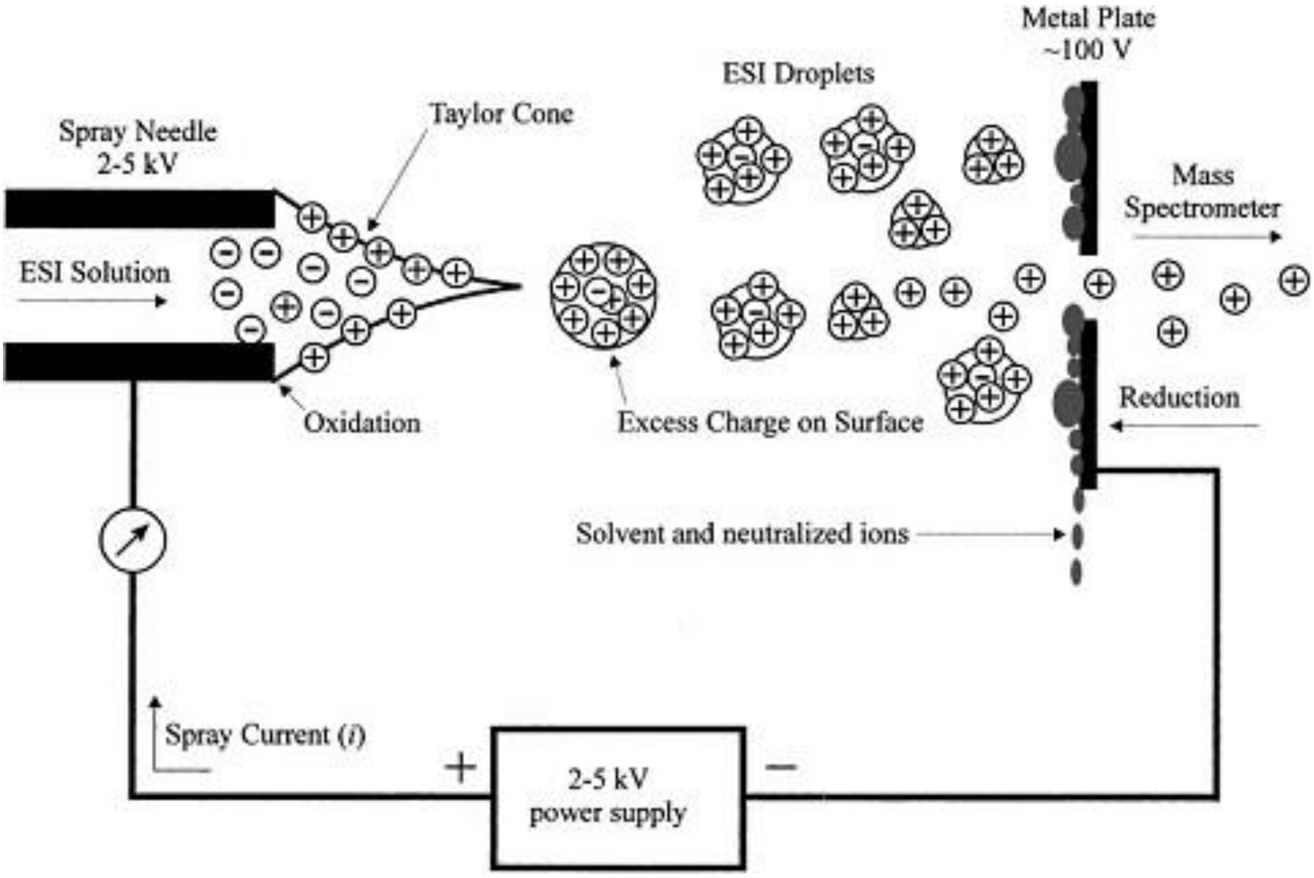


Figure 7. ESI Interface scheme.

Mass analysers have the task of separating the ions formed in the source on the basis of the mass/charge ratio (m/z).

The typology of an analyser depends on the physical principle on which the separation is based.

There are different types of analysers: single quadrupole, triple quadrupole, ion trap, TOF analyser.

The single quadrupole analyser is the most used due to its ease of use, the wide range of masses covered, the good linearity in the quantitative determination, the resolution and the quality of the spectra.

The quadrupole consists of 4 or 8 cylindrical metal bars arranged in parallel and about 20 cm long. The diametrically opposed bars are in oscillating electrical contact with each other. The electrons are accelerated by accelerator plates and the oscillating electric field causes that when a molecule, with a certain charge, enters the quadrupole, it begins to have a sinusoidal trajectory (figure 8). Depending on the oscillation frequency, only molecules with a specific mass/charge ratio will be able to travel the entire quadrupole with a sinusoidal pattern without discharging on the positive or negative electrodes. The ions that manage to get out of the tunnel will enter the detection system (photomultiplier).

The quadrupole analyser is simple, can be interfaced with other analysers, has a low cost, requires low vacuum levels and has a high scanning speed (few milliseconds) but has low resolution, poor mass accuracy and has a limited mass range.

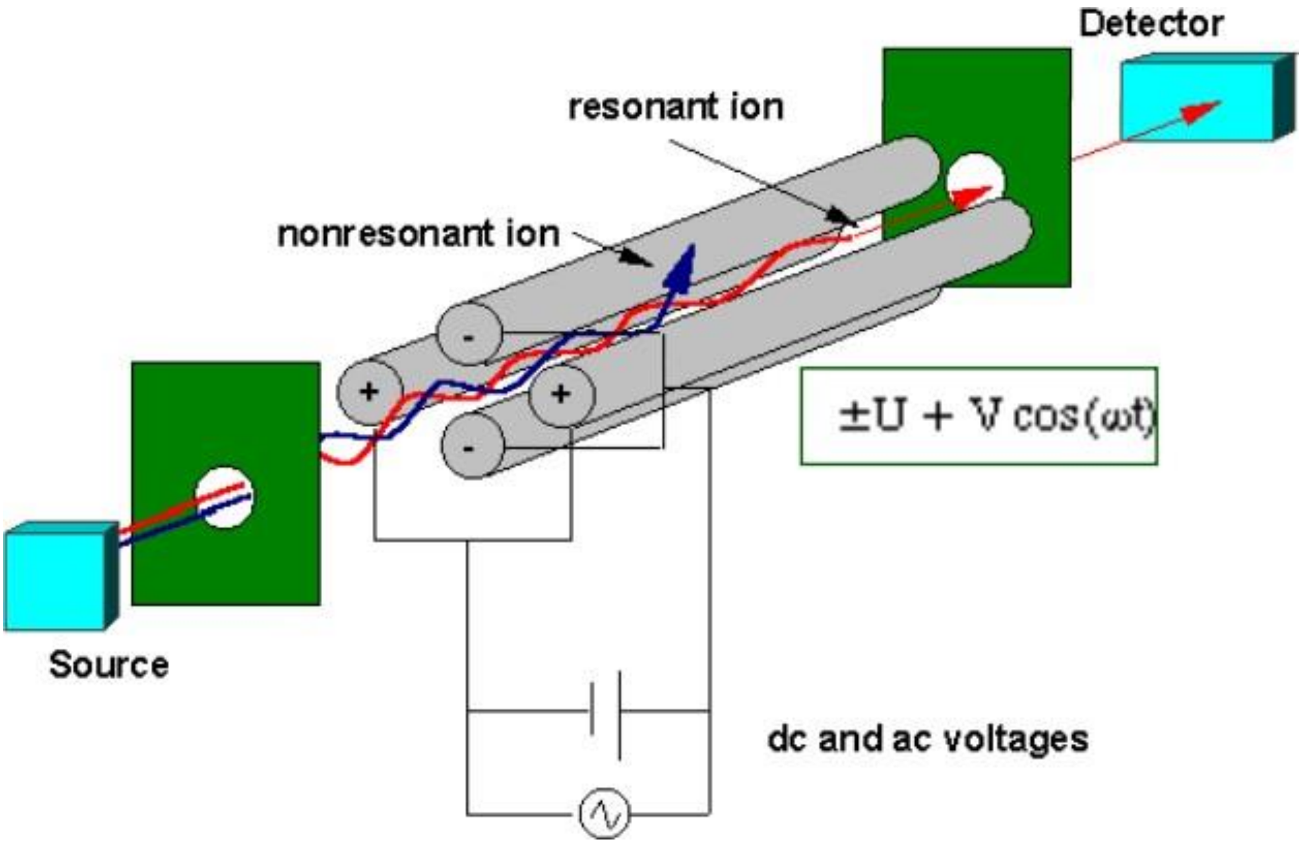


Figure 8. A single quadrupole scheme.

The triple quadrupole analyser consists of two quadrupoles in series, usually separated by a collision chamber (Q2). The latter is also a quadrupole, but regulated so as to let all the ions pass and contain a certain amount of gas (generally Argon) which causes fragmentation.

The analyser consists of a source which volatilizes and ionizes the sample; the first analyser selects ions with a certain m/z ratio (parent ion); the collision chamber in which there is a strong electric field and there is fragmentation of ions; a second analyser which measures the m/z ratio of the fragment ions produced (daughter ions); finally, a detector that serves to reveal the ions arriving in the analyser.

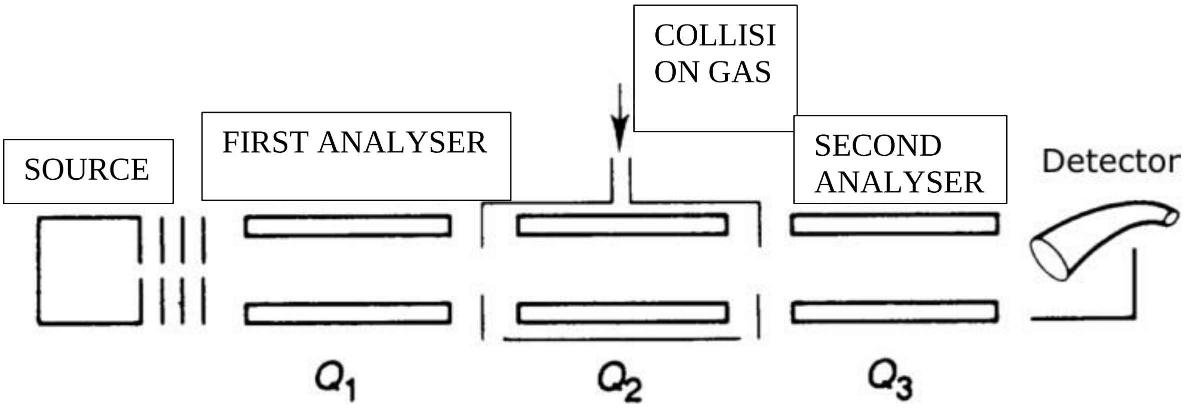


Figure 9. Triple quadrupole scheme

In the ion trap analyser, the ions are confined in a three-dimensional quadrupolar field, where the terminal electrodes control the stability of the trajectories along the z axis. To record a mass spectrum, the radio frequency of the terminal electrodes is progressively increased, causing a progressive destabilization of the gradually heavier ions which are expelled along the z axis (figure 10).

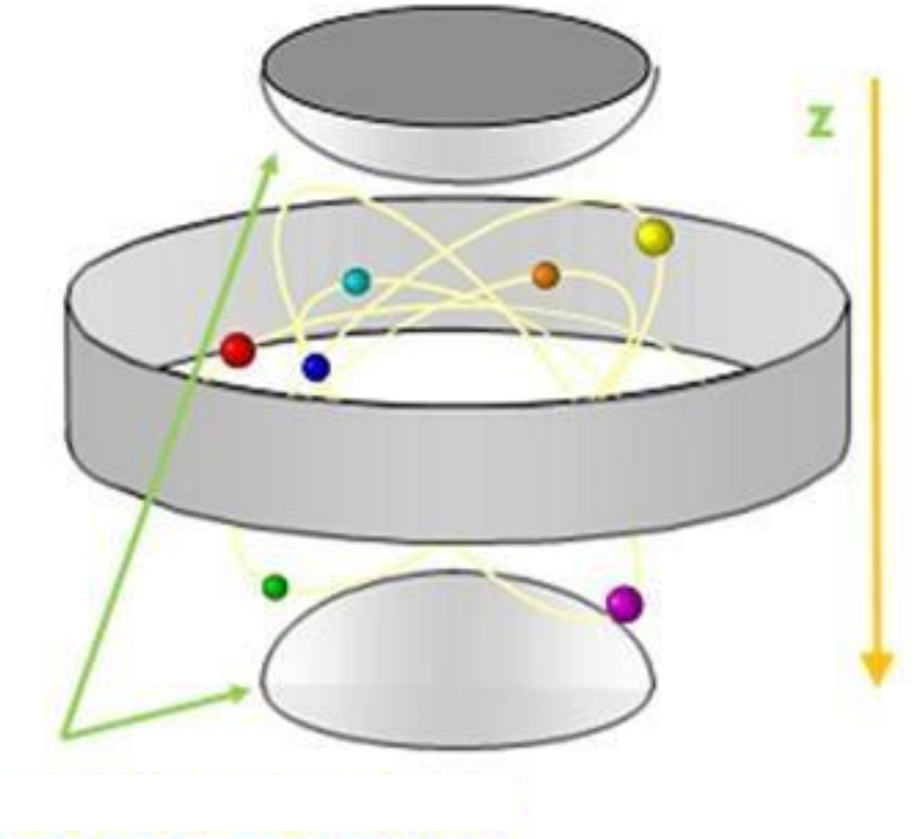


Figure 10. Ionic trap scheme.

The ion trap analyser is interfacing with other analysers, has a low cost, requires low vacuum levels, has a high scan rate and is the only analyser that allows mass experiments and tandem mass experiments but it has low resolution, poor mass accuracy (>100 ppm), limited mass range, and loss of low molecular weight fragments (<50 Da).

As anticipated, the ion trap analyser is the only analyser that allows you to perform mass experiments and tandem mass experiments. While the other analysers require the presence of multiple analysers in series in order to perform mass and tandem mass experiments.

To perform an ion trap tandem mass experiment, in the first stage of the experiment, the sample molecules from the ionizer are trapped inside the ion trap. After that, all ions are expelled, except those corresponding to the selected m/z value. Only the molecule of interest remains in the ion trap and fragmentation is caused by the application of a strong electric field. The fragments reach the detector as a function of their m/z (figure 11).

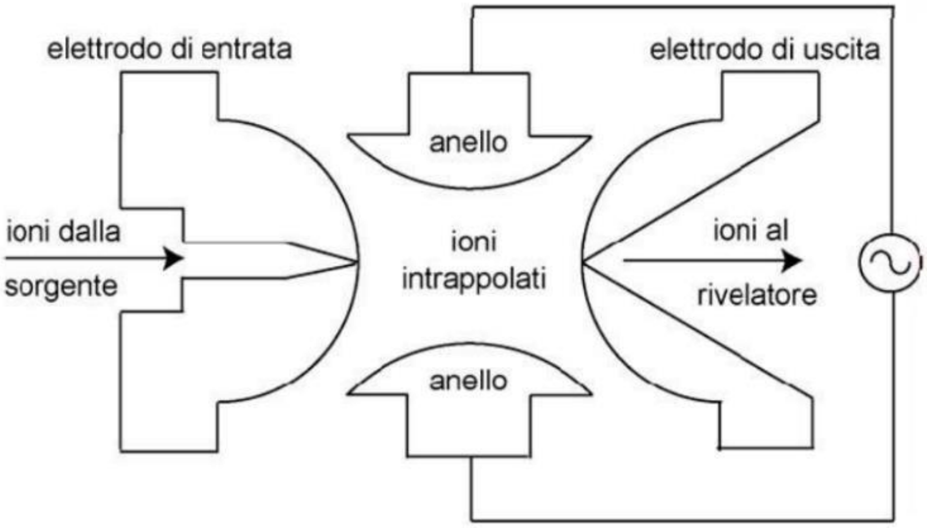


Figure 11. Ionic trap scheme

The Q-TOF analyser is a hybrid system that uses two different analysers: a quadrupole (Q) and a time of flight (TOF) analyser. TOF measures the time-of-flight it takes for a given ion to reach the tip of the analyser and then the detector. The rate at which the ions move through the analyser tube is proportional to their mass-to-charge ratio. Ions with lower m/z ratios will be accelerated more by the electric field and acquire greater velocity than ions with higher m/z ratios (Figure 12).

This analyser features high resolution, high mass accuracy (<2ppm), an extended mass range, requires high vacuum, and has a moderate scan rate.

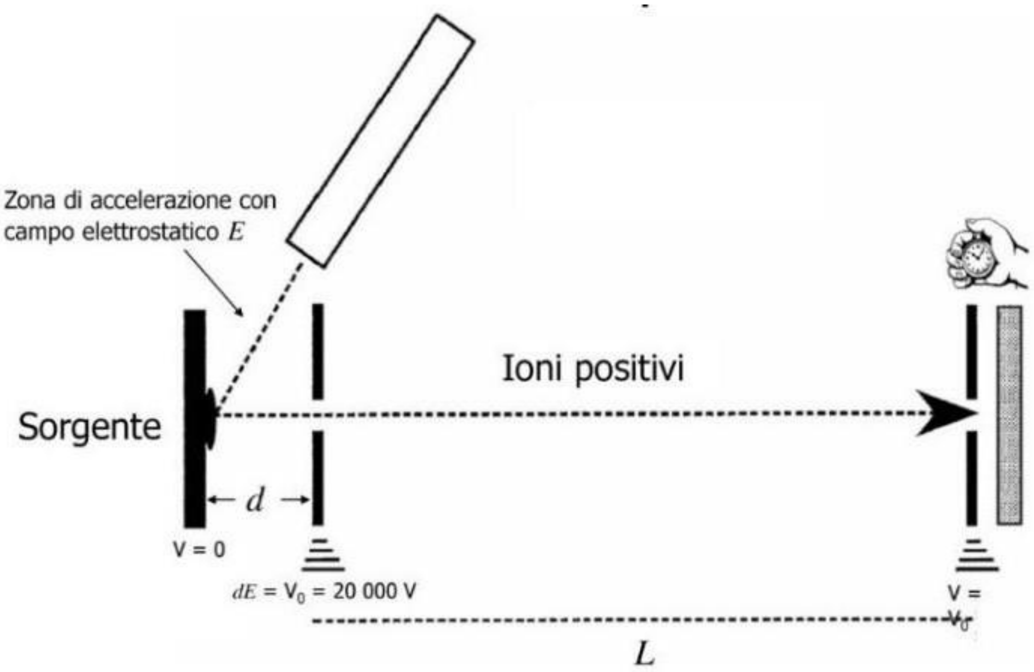


Figure 12. TOF analyser.

2. SCOPE OF THE THESIS

The aim of the thesis work, carried out at the "A. Mirri" Experimental Zooprophylactic Institute of Sicily, was to determine the presence of acrylamide in street food produced and marketed in Italy by an optimized and validated LC-MS/MS method, according to the ISO 17025/2017 standard, considering the benchmark levels established by the European Commission. This study aims to provide preliminary data on the presence of this contaminant in street food by providing initial data in order to establish a targeted control plan.

From the data obtained in the 23 types of street food products, an accurate analysis of each product was conducted, highlighting any correlations with the different cooking techniques and ingredients used. The cooking techniques necessary for the production of the various foods concern baking, frying and cooking in a pan. While as regards the composition, first of all, the primary ingredient was taken into consideration with the aim of investigating the content of asparagine and/or other free amino acids and reducing sugars present.

These comparisons were made through statistical analysis in order to highlight the parameters that influenced the concentration of acrylamide. Finally, for the types of samples that have detected significant concentrations of AA, various processing protocols for these products have been developed in order to mitigate the formation of this compound.

3. MATERIALS AND METHODS

During the thesis period, a total of 460 street food samples were taken and prepared. Details of the samples examined are in Table 2.

Tipologia di prodotto (n=20 per tipo)		Cottura
Arancine	Panelle	Fried
Cannoli	Panissa	
Crema fritta	Panzerotti	
Crocchette	Pizza fritta	
Falafel	Scagliozzi	
Courgette flowers	Seadas	
Olive ascolane	Sgabei	
Crepes	Tacos	Cooked in a pan
Dosa	Tortillas	
Piadina romagnola		
Bretzel	Erbazzone	Oven
Cecina	Fügassa	

Table 2. Details of the samples analysed.

3.1 Solvents, work solutions and instruments used

The following reagents were used for extraction and LC-MS/MS analyses: acetonitrile for LC-MS (≥99%), HPLC grade hexane (≥95%), LC-MS grade formic acid (HCOOH, ≥ 98%). The water used is bi-distilled and obtained by means of a Milli-Q® Integral 5 bi-distillation (Merck KGaA, Darmstadt, Germany).

The mobile phases used for the chromatographic run were:

1. Mobile phase A: H2O 0.1% HCOOH. The solution was prepared by adding 1 mL of HCOOH in 900 mL of water and bringing the whole to a final volume of 1 L;

2. Mobile phase B: acetonitrile 0.1% HCOOH. The solution was prepared by adding 1 mL of HCOOH to 900 mL of acetonitrile and bringing it to a final volume of 1 L.

The reference solutions of the standards used for the preparation of the calibration curve and of the fortified samples were prepared by weighing the solid reference material, taking into account its purity and, if necessary, the molecular weight. These solutions were stored in a freezer at a temperature of -20°C. From the solutions obtained (100 mg/L), by dilution the working solutions are obtained at 10 mg/L (Slz.Ref.1), 1 mg/L (Slz.Ref.2) and 0.1 mg/L (Slz.Rif.3) of acrylamide and its deuterate (Slz.Rif.Int.1 10 mg/L, Slz.Rif.Int.2 1 mg/L, Slz.Rif.Int.3 0.1 mg/ L). The solvent used was acetonitrile.

Subsequently, the working solutions were prepared, used for the extraction and for the calibration curve. The latter was adapted according to the type of sample used and the average acrylamide content found. In fact, samples with low and high acrylamide content have been identified.

In the latter case, the calibration curve was created with additional points of higher concentration (SMix5, SMix6 indicated in orange and with an asterisk).

The summary scheme is reported below:

<table>
<tr><th colspan="5">Reference solutions</th></tr>
<tr><td>Solution</td><td>Conc. (mg/L)</td><td>Weight or volume</td><td colspan="2">Final volume</td></tr>
<tr><td>Slz.Rif.1</td><td>10</td><td>-</td><td colspan="2">-</td></tr>
<tr><td>Slz. Rif.2</td><td>1</td><td>100 µL di Slz.Rif.1</td><td colspan="2">1 mL</td></tr>
<tr><td>Slz.Rif.3</td><td>0.1</td><td>100 µL di Slz.Rif.2</td><td colspan="2">1 mL</td></tr>
<tr><td>Slz.Rif.Int.1</td><td>10</td><td>-</td><td colspan="2">-</td></tr>
<tr><td>Slz.Rif.Int.2</td><td>1</td><td>100 µL di Slz.Rif.Int.1</td><td colspan="2">1 mL</td></tr>
<tr><td>Slz.Rif.Int.3</td><td>0.1</td><td>100 µL di Slz.Rif.Int.2</td><td colspan="2">1 mL</td></tr>
<tr><th colspan="5">Curve in solution</th></tr>
<tr><td>Solution</td><td>Conc. (µg/L)</td><td>Volume (µL) Siz.Rif. to add</td><td>Volume (µL) Siz.Rif.1nt3 to add</td><td>Volume (µL) of solvent to add (V. final = 1 mL)</td></tr>
<tr><td>SMix1</td><td>0,5</td><td>5 (Siz.Rif.3)</td><td>25</td><td>970</td></tr>
<tr><td>SMix2</td><td>2,5</td><td>25 (Siz.Rif.3)</td><td>25</td><td>950</td></tr>
<tr><td>SMix3</td><td>5</td><td>50 (Siz.Rif.3)</td><td>25</td><td>925</td></tr>
<tr><td>SMix4</td><td>25</td><td>25 (Siz.Rif.2)</td><td>25</td><td>950</td></tr>
<tr><td>SMix5*</td><td>50</td><td>50 (Siz.Rif.2)</td><td>25</td><td>925</td></tr>
<tr><td>SMix6*</td><td>100</td><td>100 (Siz.Rif.2)</td><td>25</td><td>875</td></tr>
<tr><th colspan="5">Sample type</th></tr>
<tr><td colspan="3">Low AA contents</td><td colspan="2">High AA contents</td></tr>
</table>

Focaccia, Piadina romagnola, Crepes	Bretzel, Arancina, Crocchette, Panelle, Panissa, Pizza fritta, Cecina, Erbazzone, Sgabei, Olive ascolane, Crema fritta, Scagliozzi, Seadas, Dosa, Falafel, Tacos, Tortillas, Cannoli, Courgette flowers

Table 3. Work solution, used for extraction and the calibration curve.

3.1 Extraction of the samples

The extraction is the procedure that allows to isolate the analyte of interest from the mass of the matrix. It is generally followed by a purification step to remove any co-extracted interferents, although often the extraction and purification step coincide in a single step [42].

Extraction conditions must be optimized to maximize analyte recovery. In this regard, recovery tests must always be carried out with certified material or with samples fortified with an appropriate concentration of standard mixture.

One of the best known and most used extraction techniques is SPE (Solid Phase Extraction) extraction, initially introduced as an alternative to liquid-liquid extraction. The extraction process is based on the interaction of the analytes to be extracted dissolved in a liquid or sometimes gaseous phase with a solid phase (adsorbent). After preliminary conditioning of the adsorbent, the extraction process generally involves a phase of loading of the liquid sample (or passage of the gaseous sample) and retention of the analytes, followed by an elution phase with a suitable solvent.

SPE exploits the same analyte-adsorbent interactions used in the HPLC separation technique but the objective is not to separate the analytes on the basis of their different affinity towards the stationary phase and the liquid phase, but to strongly retain the analyte or a class of analytes during sample loading, then elute them completely, with the smallest possible volume of solvent. Furthermore, compared to an HPLC column, an SPE column has a much lower number of theoretical plates (N) (about 20).

This technique allows to considerably reduce the consumption of solvents and to obtain higher concentration factors and highly purified extracts. It requires shorter extraction times, is relatively inexpensive, can be easily automated and interfaced with chromatographic or spectroscopic separation techniques.

The dispersive solid-phase extraction (dSPE) is a variant of the classic SPE which involves putting the liquid sample in contact with an adsorbent phase, or a combination of several phases, capable of selectively retaining some interferents. dSPE is an integral part of a sample extraction and purification technique known as QuEChERS.

For the extraction of acrylamide, dispersive solid phase extraction with the use of QuEChERS was used.

QuEChERS is the acronym of quick, easy, cheap, effective, rugged and safe, i.e. rapid extraction techniques born for the analysis of pesticides in plant samples but today are used for many determinations.

This technique represents an interesting alternative to classic liquid-liquid extraction and solid-phase extraction, as it saves time and solvents.

The process consists of two steps, a solvent extraction step followed by dSPE to remove co-extracted interfering substances.

In the first step, i.e. the solvent extraction step, the sample is homogenized and extracted with a water-miscible organic solvent (acetonitrile), in the presence of large amounts of salts (NaCl, MgSO4), acids and buffers (citrate buffer) to improve phase separation, extraction efficiency and protect analytes that may be degraded.

In the second part there is the transfer of a portion of the organic extract into a PP tube containing a dehydrating agent, to remove traces of water, and a combination of adsorbents, to remove unwanted components.

The commonly used salts are magnesium sulfate (MgSO4) and sodium chloride (NaCl) in the ratio of 2:1 or 4:1 [43].

All contain PSA (primary and secondary amine) as the adsorbent phase to remove sugars and fatty acids, and magnesium sulfate as a dehydrating agent. There may also be tubes filled with other sorbents to improve sample purification (such as graphitized carbon to remove pigments and sterols and C18-phase to remove non-polar interferents such as lipids).

The extraction of the samples examined in this work was according to the following:

1. 1.0 ± 0.1 g was accurately weighed directly into a 50 mL centrifuge tube;
2. 25 µL of the Slz solution were added. MRlnt at a concentration of 1 mg/L to all samples (25 µg/kg in matrix — 2.5 µg/L in solution);
3. 5 mL of H2O were added and in the presence of fried products hexane was added to degrease (5 mL);
4. The solution was vortexed for about 1 minute then, 10 mL of Acetonitrile for LC-MS/MS were added. Samples were vortexed for 1 minute;
5. a mixture of salts containing 4g of MgSO4, 1g of NaCl, 0.5g of sodium citrate dibasic sodium citrate sesquihydrate, 1g of sodium citrate tribasic dihydrate was added;
6. the samples were vortexed for 1 minute and then centrifuged for 5 minutes at 3500 rpm;

7. The centrifugated were filtered again through 0.45 µm filters and 1 mL of filtrate were transferred into 2 mL vials and injected into LC-MS/MS.

Hexane has always been used in fried products to remove oil residues, since it is a selective aprotic apolar solvent for fatty substances and which has a strong degreasing power.

Hexane was not used in baked and pans goods as the amount of oil used was minimal or often absent.

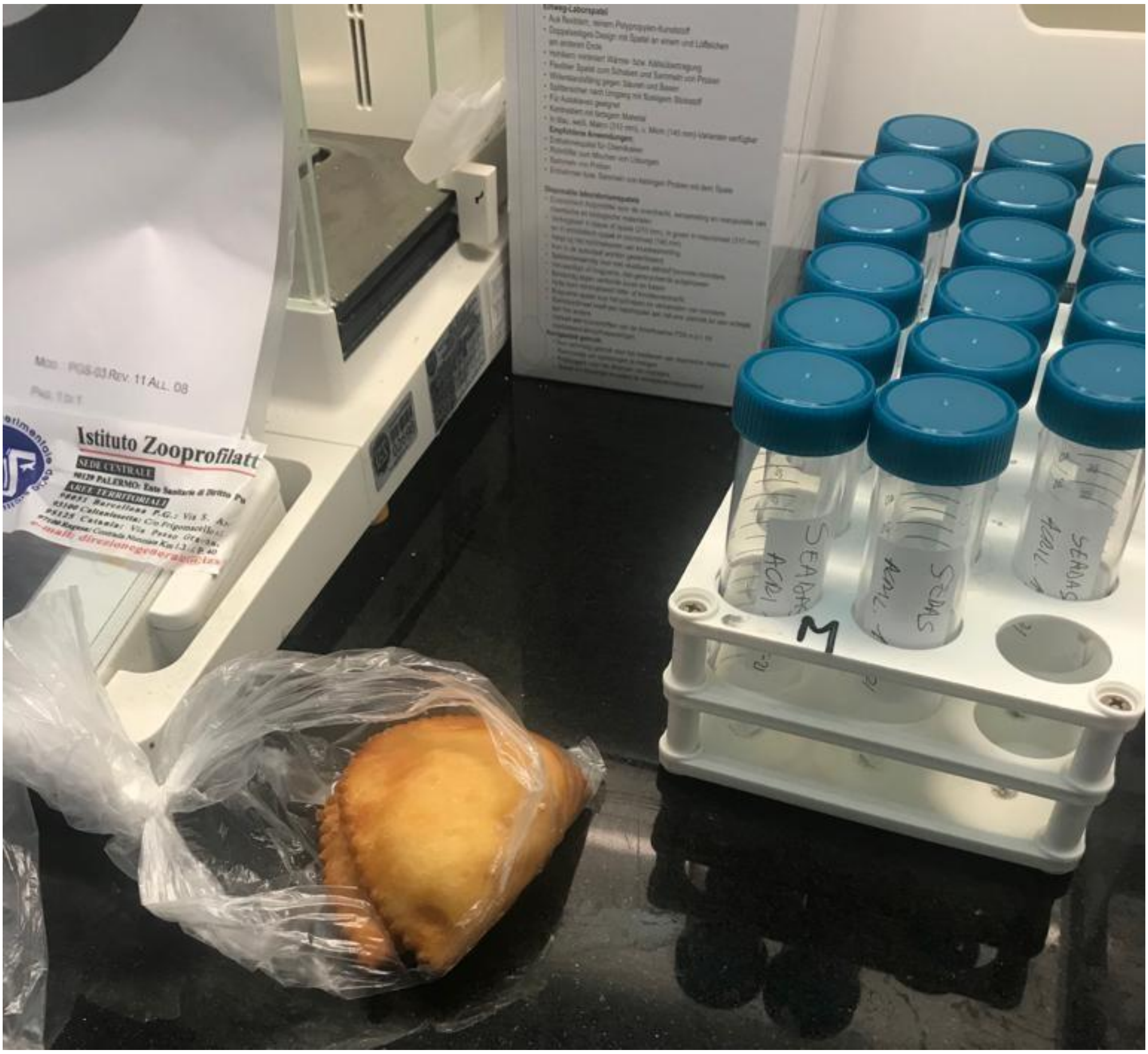


Figure 13. Weighing of the samples.

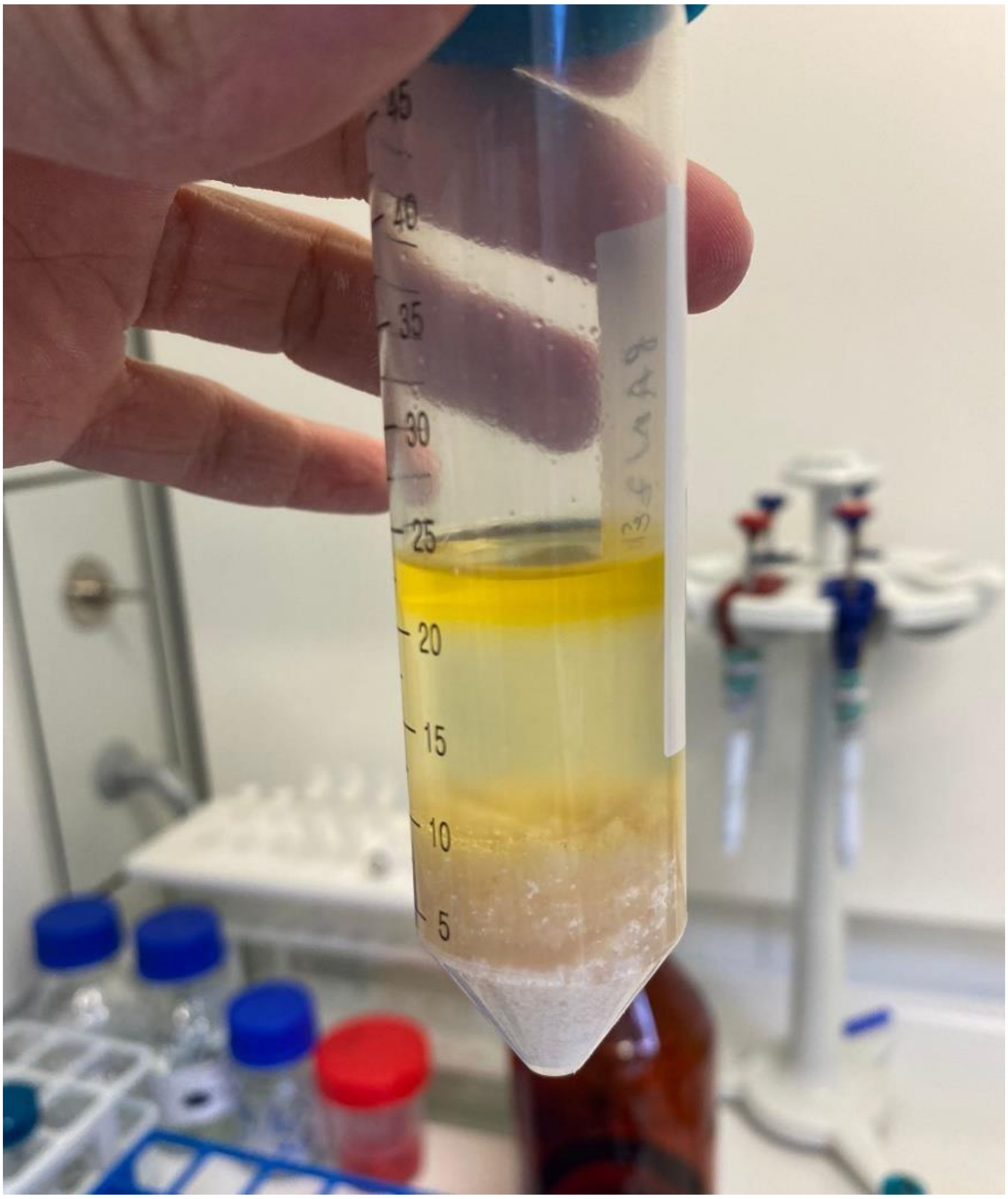


Figure 14. Extraction by QuEChERS method.

3.2 UHPLC analysis

The analysis were performed in a Thermo Fisher UHPLC system (Thermo Fischer Scientific, California, U.S.A.) consisting of an ACCELA 1250 LC pump and an ACCELA autosampler (Thermo Fisher Scientific, California, U.S.A.). Chromatographic separation was achieved using a 3 µm - 2.7x150 mm Ascentis Express C18 column. The flow was maintained at 0.3 mL per minute, the autosampler temperature was 6°C, the column was maintained at a temperature of 30°C. The injection volume was 25 µL.

The mobile phases, phase A ($H2O$ 0.1% HCOOH) and phase B ($H2O$ 0.1% HCOOH) were maintained in a ratio of 98:2 throughout the chromatographic run with a total duration of 5 minutes.

Figure 16. UHPLC instrumentation used.

The detector used is a triple quadrupole TSQ Vantage (Thermo Fisher Scientific, California, USA). The modality used was positive electrospray ionisation (ESI+). The ESI parameters were: capillary voltage 3.0 kV, capillary temperature 350 °C, vaporiser temperature 200 °C. The sheath Gas Pressure was set at 30 while the Auxilary Gas Pressure 15. The product ions were obtained by direct infusion of an acrylamide solution at a concentration of 1 mg/L with a flow rate of 10 μL/min.
The characteristic ions obtained are shown below:

Molecule	Parent	Product (CE)	Q1	Q3
Acrylamide	72,0	27,0 (18)	0,7	0,7
		55,0 (10)	0,7	0,7
Acrylamide d-3	75	30,0 (18)	0,7	0,7
		58,0 (10)	0,7	0,7

Table 4. Characteristic ions of acrylamide and its deuterate.

The values indicated in brackets indicate the collision energy (CE) used to obtain the indicated fragmentation.

The analysis sequence involved a first injection of blankeluent (acetonitrile) and subsequently the injection of calibration curve concentration points. These were chosen on the basis of the acrylamide values found.

For low concentration samples, the analysis sequence was:

1. eluent blank;
2. SMix 1 (0.5 µg/L);
3. eluent blank;
4. Smix3 (5.0 µg/L);
5. eluent blank;
6. fortified matrix blank (see paragraph 3.4.1);
7. eluent blank;
8. sample to be analysed;
9. eluent blank.

For the high concentration samples the points in the sequence were SMix 3 (5.0 µg/L) instead of SMix1, and SMix 5 (50 µg/L) instead of SMix 3.

To define the values found acceptable, the difference between Smix3 obtained from the first set and that of Smix3 obtained from the second set must be less than or at most equal to 5%, if not, the calibration curve was repeated. It was also verified that the QC blank does not contain any signals attributable to the analyte (signals < the LOD or < 10% of the lowest point of the calibration curve). If each of the previous parameters was not respected, the tests were repeated eliminating possible random errors evaluated with internal staff.

For the identification, the following formula was used: TR(c)=TR(a)±0.1; where TR(c) represents the retention time related to the analyte in the test sample, while TR(a) refers to the retention time related to the analyte in the fortified sample.

The sample is considered positive when the S/N ratio of the lowest intensity ion fragment is ≥ 3. For quantification, only the contribution of the ion or transition with the highest intensity was considered. The result has been corrected by recovery.

Fortified samples are obtained by adding an exact volume of the Slz.Rif.MR solution to an aliquot of test sample, as shown in Table 5.

Spiked samples	**Concentration in matrix (µg/Kg)**	**Volume of Slz.Rif. to collect**
Crunchy products	300 µg/Kg	30 µL a 10 mg/L (Siz.Rif.1)
Soft baked goods	400 µg/Kg	40 µL a 10 mg/L (Siz.Rif.2)
Fried potato products	750 µg/Kg	50 µL a 10 mg/L (Siz.Rif. l)

Table 5. Spiked samples preparation scheme.

3.3 Application of the method in real samples

The method was used on real samples in order to detect the presence of acrylamide.

The test and measurement equipments were calibrated and reference materials certified by external companies were used for method validation which were subsequently stored according to the methods indicated on the packages or in the relative certificates of analysis.

The real samples were bought in various fry shops in Palermo and/or supermarkets. Some of them have been produced in laboratories when not available through these channels. The products under study were baked, fried or pan-cooked products marketed in Italy.

Figure 16. Some of the prepared and analysed street food products.

Figure 17. Some of the prepared and analysed street food products.

Figure 18. Some of the prepared and analysed street food products.

3.4 Method validation

The validation of an analytical method is the process through which it is established, through laboratory studies, that the characteristic performance of the method is adequate for the intended application.

The standard that regulates the validation process of an analytical method, through a series of precisely defined experiments and measurements in the context of a test or calibration laboratory, has the reference code ISO/IEC 17025. This standard is consisting of management requirements and technical requirements [42].

The management requirements concern the organization, the quality system, the control of non-compliant activities, including corrective and preventive measures, and finally the internal audits. Instead, as regards the technical requirements, all the factors that can affect the correctness and reliability of the tests performed are studied, such as: sampling, personnel, workplace, environmental conditions, test methods and their validation, equipment, traceability of the measures, guarantee of the results and therefore their presentation.

The ISO 17025 standard also mentions the criteria to be used for the calculation and the expression of the measurement uncertainty, which must be obtained whenever possible.

In order to meet the verification requirements of the ISO 17025 standard, the European Community decision 657/2002/EC constitutes a real technical guide for the development of analytical methods [44]. The decision establishes the rules for the analytical methods used to analyse the official samples, respects the instructions contained therein and sets out the general standards for the interpretation of the results of the controlled official laboratories.

In general, whenever a non-standardized test method or procedure, or a method developed and designed by a laboratory (in-house method), is used, the analytical method must be verified.

According to the standard, the estimate of measurement uncertainty is extremely important, i.e. the parameter relating to the result of a measurement, which characterizes the degree of dispersion of the value reasonably attributable to the result.

Quantitatively, a series of parameters must be formulated, as specified by ISO17025:2018 and by EC Decision 657/2002, which are defined as follows:

- range of linearity: range of analyte concentration for which the instrumental response is directly proportional to the concentration;
- calibration curve: curve obtained by injecting increasing concentrations of the analyte dissolved in the solvent;
- repeatability: degree of agreement between a series of measurements of the same measurand, when the single measurements are carried out leaving the measurement conditions unchanged. Conditions of repeatability are the conditions under which independent test results are obtained with the same method on identical test items in the same laboratory and with the same operator using the same equipment;
- accuracy: expresses the degree of agreement between a single measured value and the true value of the measurand;
- percentage recovery: percentage of the actual concentration of a substance recovered during the analytical procedure;
- limit of detection (LOD): the minimum measured concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty;
- limit of quantification (LOQ): the lowest analyte content that can be measured with reasonable statistical certainty;
- robustness: the ability of the method to be not significantly influenced by variations in the analytical conditions. This parameter serves to qualify the reliability of a procedure during its routine use or the possibility of reproducing the analytical method in different laboratories and at different times, without a significant difference in the results;

- accuracy: indicates the proximity of the average of the values obtained to the true or accepted as true value;

Intra-laboratory reproducibility can be measured within the same laboratory, with the same method, on the same test material, by different operators, instruments, at relatively long time intervals;

- specificity: ability of a method to distinguish between the analyte to be measured and other substances;
- measurement uncertainty: it is a parameter associated with the result of a measurement that characterizes the dispersion of the values that can reasonably be assigned to the measurand.

For the validation of the method, the specificity, linearity range, repeatability and accuracy by percentage recovery, the limit of detection (LOD), the quantitative limit of detection (LOQ), the expanded uncertainty, the robustness and accuracy were measured [45].

This was verified by analysing 20 blank samples by product type in order to verify the absence of interfering peaks at the acrylamide retention time.

The range of linearity was calculated by constructing a calibration curve in solution with increasing concentration of the analyte. 7 concentration points were used including zero (0-0.5-2.5-5-25-50-100 µg/L). These concentrations correspond in the matrix to 5-25-250-500-100 µL/kg (dilution factor 10). The internal standard was used at a concentration of 25 µg/L.

The repeatability, expressed as a relative standard deviation or relative standard deviation (RSD) and the accuracy expressed as a percentage recovery, were calculated by the determination of acrylamide in samples fortified at three levels (150-300-450 µg/kg), 10 samples for each level.

Recovery is expressed as the ratio between the analyte concentration measured at the end of the analytical process and that added initially. It was determined during validation by quantification with external standardization.

$$\text{Recovery (\%)} = \frac{C1 - C2}{C3} * 100$$

Where C1: analyte concentration measured after addition C2: analyte concentration measured before addition C3: concentration added.

The LOD and LOQ were calculated on the basis of the analysis of 20 blanks by type of matrix (soft bread, crispy bread and potato-based foods). The equations used for the calculation were:

LOD = (3.9 • SDB)/p LOQ= 3.3 • LOD

Where SDB is the standard deviation of the response (area) of the blank and p is the slope of the calibration curve.

The uncertainty was calculated with a coverage factor of 2 corresponding to a level of confidence of approximately 95% for the three levels of fortification low (150 µg/kg), medium (300 µg/kg) and high (450 µg/kg).

For all experimental conditions which may be subject to oscillation in practice (e.g. stability of the reagents, sample composition, pH, temperature) any variation which may influence the result of the analysis must be indicated.

3.5 Statistical analysis

The statistical analysis were conducted using the R 4.1.1 software and the following qualitative variables were identified:

- sample;
- presence or absence of acrylamide: detectable or not;
- type of cooking: fried, oven, pan;
- type of flour used: chickpeas, whole chickpeas, 00 flour, 00 flour and 0 flour, 00 and manitoba flour, manitoba flour, durum wheat flour, corn, absent, potatoes, rice and lentils;
- type of oil used: peanut, sunflower, extra virgin, absent.

Normality was tested for the quantitative variable acrylamide using the Shapiro-Wilk test. The distribution was not normal (p-value<2.2e-16). The difference between the various samples was then evaluated using the Kruskal-Wallis test.

As regards the types of flour, there is no statistically significant difference between flours of the same type. I therefore identified: wheat, chickpeas, corn, rice and lentils, potatoes.

The results obtained are shown in Table 6:

Group	**Response variable**	**p-value**
Cooking	Acrylamide	1,034e-4
Flour	Acrylamide	<2,2e-16
Oil	Acrylamide	0,6895

Table 6. Kruskal-Wallis test results.

The difference between cooking methods (oven, pan and fried) and between types of flour was tested using the Siegel and Castellan test [46].

4. RESULTS AND DISCUSSION

4.1 AA contents in the street food analysed

The validation values and categories of the various products analysed are shown in table 7.

Matrix	Concentration levels	Linearity µg/L	Measure range	LOD	LOQ	Mean recovery	Repeatability	Expanded uncertainty
Crunchy bread	150	5-1000	30-40	3,9	13	102%	58	25,2
	300						54	27,0
	450						85	41,7
Soft bread	20	5-1000	5-60	0,8	3	98%	4,3	4,0
	40						9,5	6,0
	60						1,3	7,5
Fried potato products	250	5-1000	30-750	8	26	102%	70	31
	500						65	33
	750						24	25
Products that fall like crusty bread			Bretzel, Cecina, Dosa, Erbazzone, Fügassa, Tacos					
Products that fall like soft bread			Crepes, Piadine, Tortillas					
Products that fall as fried potato products			Arancine, Cannolo, Crema fritta, Crocchette, Falafel, Fiori di zucca, Olive ascolane, Panelle, Panissa, Panzerotto, Pizza fritta, Scagliozzi, Seadas, Sgabei					

Table 7. Validation values and product categories (concentration level, measuring range, LOD, LOQ, repeatability and expanded uncertainty are expressed as µg/kg).

76% of all the analysed samples revealed acrylamide contents according to the developed method (samples with acrylamide contents > LOD). The percentage of AA found above the LOD was 100% in pretzels, cannoli, fried cream, crepes, croquettes, dosa, falafel, courgette flowers, panzerotti, wraps, seadas and tortillas; 95% (19 samples) in cecina, erbazzone and fügassa; 80% (16 samples) stools; 70% (14 samples) stuffed olives; 35% (7 samples) panissa and scagliozzi; 25% (5 samples) arancini and 10% (2 samples) panelle. In fried pizza and tacos, the percentage of detectable acrylamide is equal to 0%; none of the samples, therefore, detected concentrations of acrylamide above the determination limit of the method developed (Table 9).

	Type of products	**Mean value (µg/kg)**
Oven cooking	Bretzel	192.32 ± 76.17
	Erbazzone	69.14 ± 50.42
	Cecina	25.44 ± 6.34
	Fügassa	22.78 ± 8.27
Frying	Courgette flowers	286.93 ± 345.19
	Crocchette	232.99 ± 145.10
	Panzerotti	94.31 ± 13.43
	Cannoli	63.89 ± 13.95
	Seadas	62.04 ± 24.71
	Falafel	55.55 ± 6.62
	Olive ascolane	51.32 ± 31.14
	Crema fritta	24.75 ± 8.04
	Scagliozzi	24.13 ± 22.96
	Sgabei	14.08 ± 4.55
	Arancine	11.48 ± 7.26
	Panelle	9.06 ± 3.33
	Panissa	8.67 ± 1.07
	Pizza fritta	8.00 ± 0.00
Pan cooking	Piadine	83.28 ± 90.02
	Dosa	46.28 ± 11.98
	Tortillas	39.02 ± 22.58
	Crepes	18.52 ± 4.48
	Tacos	3.90 ± 0.00

Table 8. Mean acrylamide values in descending order grouped by cooking condition.

The highest value of acrylamide content was found in a sample of courgette flowers, equal to 1539.8 µg/kg while the lowest value was detected in a sample of panissa, equal to 8.3 µg/kg.

Considering the different types of cooking, we verified a higher incidence of positive samples (with acrylamide contents higher than the LOD) in oven-cooked products (96.3%), followed by pan-cooked products (73%) and fried products (68.2%).

As regards the average values, the baked samples revealed the highest average acrylamide contents (77.67 ± 83.01 µg/kg), followed by the fried products (67.65 ± 129.12 µg/kg) and those cooked in a pan (38.20 ± 49.24 µg/kg).

Statistical analysis revealed significant differences in acrylamide concentration between different types of products (Kruskal-Wallis chi-squared = 351.42, df = 22, p-value < 2.2e-16). The post-hoc multiple comparison test by Siegel and Castellan (1988) [46] determined which data groups contributed most to the statistical difference. The aforementioned test revealed that tacos, pretzels, nuggets, and courgette flowers were the data groups that contributed the most to the significant difference.

The difference in the results arises from the plurality of products and therefore of ingredients used for the preparation of the samples under study. These play an important role in acrylamide formation since different ingredients are characterized by different amounts of asparagine and/or other free amino acids and reducing sugars.

Considering this, I decided to check any significant differences between different primary ingredients of each product type.

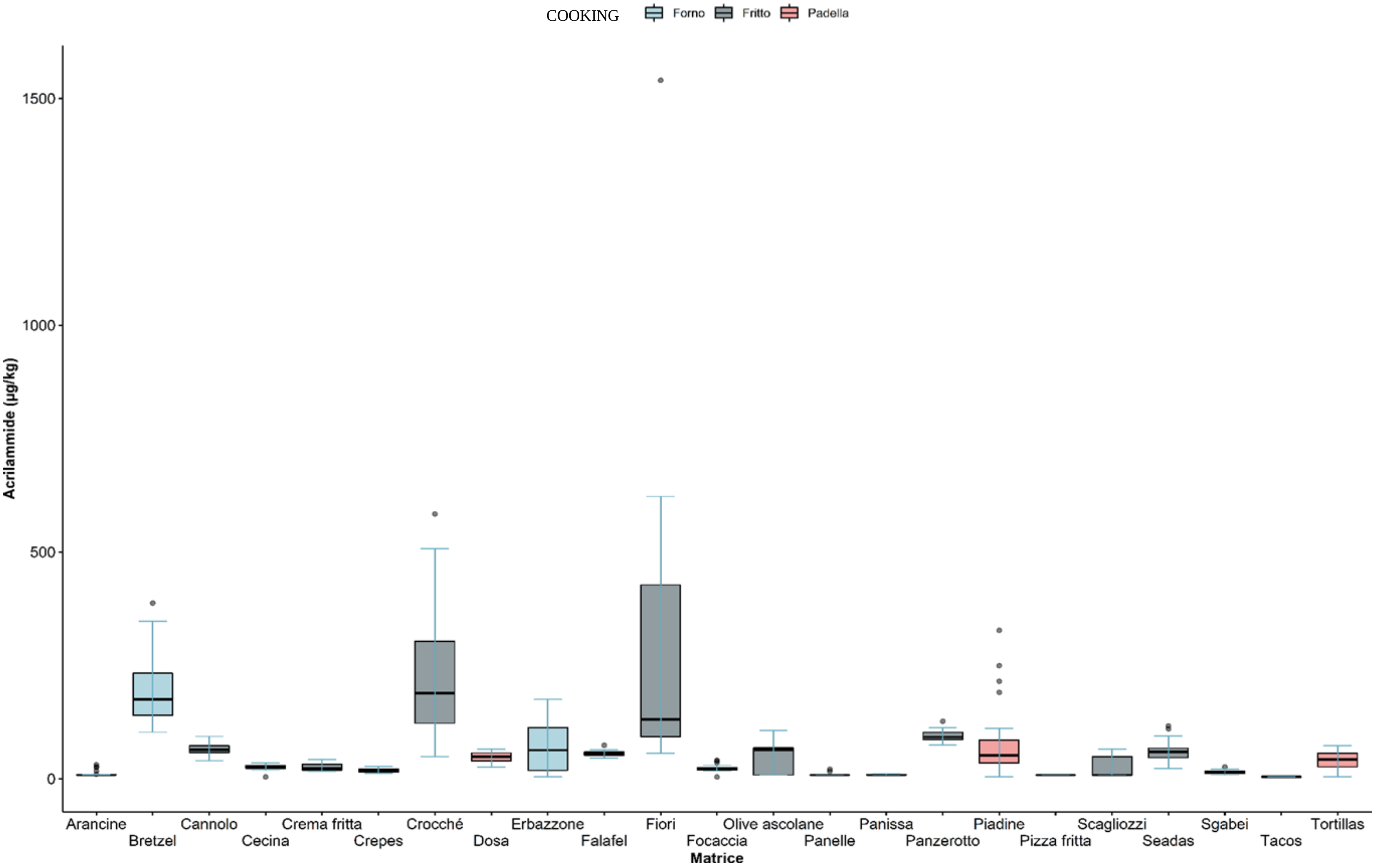


Figure 19. Comparison of acrylamide concentration sorted by product type. Fiori = cougette flowers.

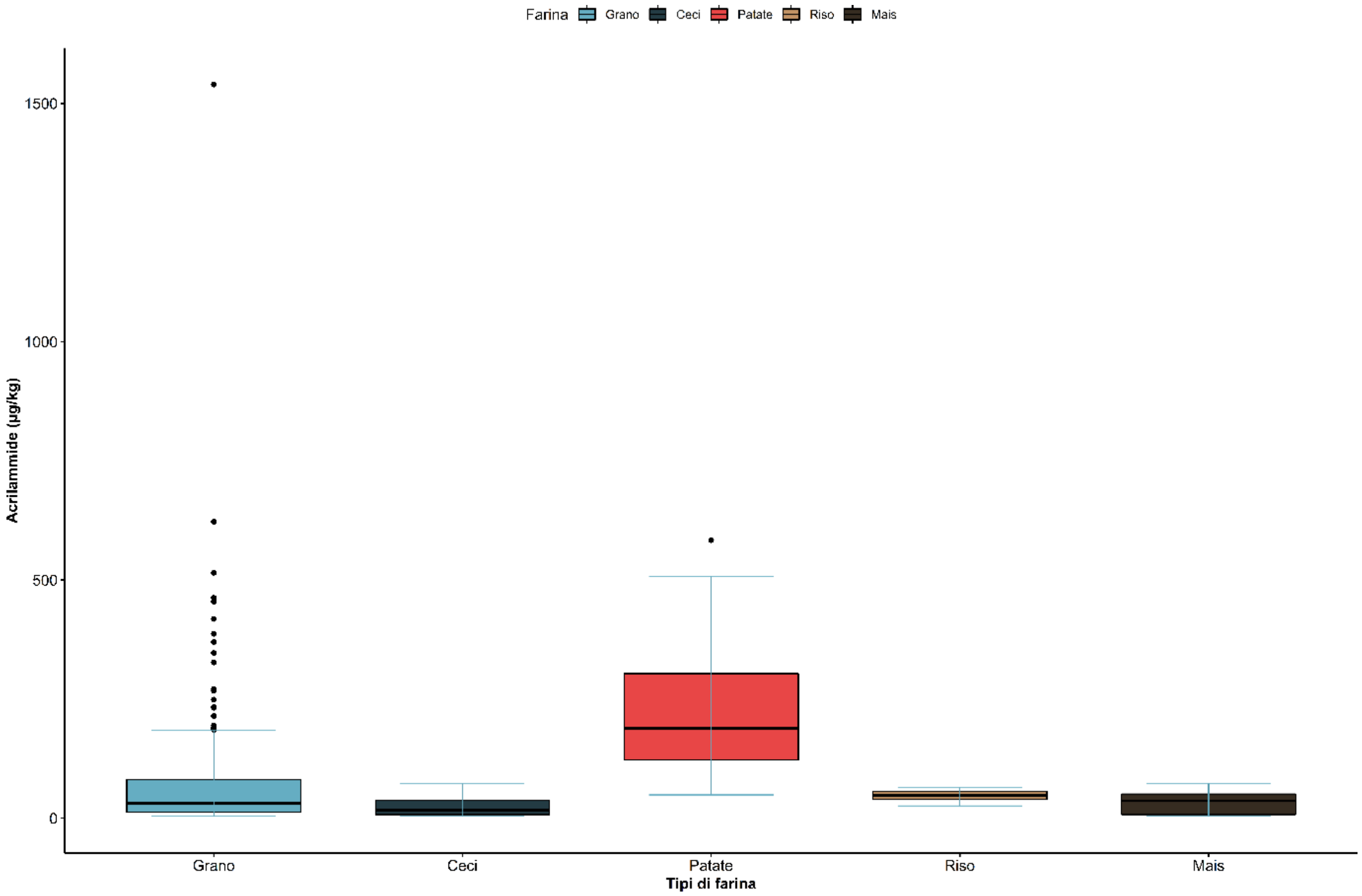


Figure 20. Comparison of flour types and acrylamide concentration. Grano = wheat; Ceci = chickpea; Patate = potatoes; Riso = rice.

Figure 20 represents the comparison between the different flours used. In this regard, the statistical analysis carried out revealed a significant difference between flours of the same cereal (durum wheat flour, 00, 0, manitoba) or legumes (for example whole chickpeas, chickpea flour etc.; Kruskal-Wallis chi- squared = 65.327, df = 5, p-value = 9.585e-13).

The Kruskal-Wallis test followed by the post-hoc test by Siegel and Castellan (1988) showed significant differences between different types of flour (wheat, chickpeas, corn, rice).

The statistical analysis conducted by type of cooking verified a significant difference (Kruskal-Wallis chi-squared = 18.355, df = 2, p-value = 1.034e-4) whereas the type of oil used for cooking had no influence on the difference between groups of fried samples (Kruskal-Wallis chi-squared = 1.4689, df = 3, p-value = 0.6895).

Regarding the fried samples, the high concentration of acrylamide in courgette flowers can be attributed to the batter, a technique widely used in the preparation of fried foods such as vegetables, fish or fruit. There are several recipes but flour is the essential element accompanied by water, milk or eggs [7].

In this specific case, the recipe for the preparation of the batter involved the use of soft wheat flour, water, salt and yeast.

The wheat flour that coats the products can be the cause of acrylamide formation as it contains the key factor, i.e. free asparagine with values of 1.74-19.05 mg/100 g [47]. The asparagine content may vary according to the different cereal varieties.

In addition to asparagine and carbonyl compounds, acrylamide formation can be influenced by other amino acids that can increase it (glutamine, lysine, alanine, threonine) or decrease it (glycine, proline, cysteine) [48].

Consultation of previous studies showed that the batter obtained with wheat flour determines higher levels of acrylamide than the wheat-corn flour, rice flour and chickpea flour. Furthermore, the highest acrylamide content was obtained with the flour-water mixture, followed by flour-milk and flour-egg [49].

Indeed, the data obtained from courgette flowers in batter seem to be in line with what has been said; in fact, wheat flour mixed with water was used and quite high values were recorded.

It seems that acrylamide formation is also related to oil absorption of fried batter but no correlation has been found in previous studies [50]. In addition to courgette flowers, croquettes, a typical Neapolitan product based on potatoes with a soft and stringy heart of provola or mozzarella, have reported high acrylamide values.

The formation of acrylamide in the croquettes is a consequence of the presence, in the potatoes, of available reducing sugars, in particular glucose and fructose, united by the frying conditions (time and temperature). Reducing sugars are present in higher quantities than free asparagine [51]. However, reducing sugar levels in potatoes vary widely by variety, depending also on soil condition, soil fertilization, location, and weather conditions [52].

The main pathway of acrylamide formation is the Maillard reaction with free asparagine as a precursor [22]. However, the mere presence of asparagine results in limited acrylamide formation but when a carbonyl source is present in the food, the acrylamide yield is higher, as asparagine needs a carbonyl to accelerate the conversion to acrylamide. This would explain the high level of acrylamide found in foods rich in reducing sugars and free asparagine such as potato and fried products [53].

The cooking temperature and time play a fundamental role; in fact, the formation of acrylamide begins at temperatures above 120 °C and reaches a maximum at 170-180 °C [7]. In addition to the cooking temperature, the formation of acrylamide is also related to the storage temperature of potato tubers.

In fact, storing potato tubers below 8°C leads to the phenomenon called "low temperature sweetening" which causes an increase in the reducing sugar content and an increase in the brown pigment during frying and therefore higher quantities of acrylamide. To avoid the increasing of the reducing sugar content, potato tubers should be stored at a temperature between 8°C and 12°C [25].

Serving size is another factor that can contribute to acrylamide variation as it affects cooking time; this may explain the different concentrations obtained in the 20 kibble samples.

The croquettes were fried in peanut oil but the type of oil used in the frying process has no influence on the formation of acrylamide, as verified by the statistical analysis reported above.

Another product that revealed high concentrations of acrylamide is bretzel, a particular bread in the shape of a knotted ring which is prepared according to the bread-making method called Laugengebäck. In Germany it indicates bread which, before baking, is dipped for a few seconds in a boiling solution of water and caustic soda (sodium hydroxide). The soda gives the particular glossy aspect characteristic of this product and loses its causticity during cooking in the oven. However, caustic soda is used almost exclusively in industrial preparations, while in the home it is common to use sodium bicarbonate to avoid the risks deriving from the manipulation of sodium hydroxide. In German-speaking countries the use of caustic soda is more common.

Pretzels fall into the crispy bread category with reference values of 350 µg/kg according to EU Regulation 2158/2017 (Table 9). Only one sample exceeds the reference levels. However, given the heterogeneity of the products analysed, difficulties were encountered in assigning the product category according to EU Reg. 2158/2017, critical issues that would lead us to reflect on a possible scenario of expansion of the products considered by this regulation. For these reasons, we have made an assignment on a subjective basis mainly linked to the type of cooking and the primary ingredient used.

The ingredients used were wheat flour, water, butter, baking powder, baking soda, salt and sugar. By making a careful analysis of the ingredients, it can be noted that there is the presence of wheat flour which has a high content of free asparagine, responsible for the formation of acrylamide. However, there is also the presence of baking powder, baking soda and salt in the outer surface (crust) as well.

These ingredients appear to be limiting agents of acrylamide formation but this is not supported by the results obtained. Yeast seems to reduce the formation of acrylamide as it uses asparagine for its metabolic activity, reducing its presence [7].

In the case of pretzels, this activity may not take place as the leavening time is very short (30 minutes). Therefore, the effect of the yeast is negligible due to the reduced leavening times.

Sodium bicarbonate (NaHCO3) is a sodium salt of carbonic acid used as a chemical leavening agent that decomposes into carbon dioxide when exposed to heat [7].

It is a food additive, coded with the abbreviation E 500.

In a comparison study with ammonium bicarbonate, sodium bicarbonate caused the reduction of the acrylamide content due to the increase in pH.

In biscuits, for example, it resulted in a 70% reduction in the acrylamide content [54]. In the presence of ammonium bicarbonate, the increase of acrylamide is predictable because it provides more reactive carbonyl compounds such as glyoxal and methylglyoxal originating from the reaction of ammonia with reducing sugars [55].

Thus, the increase in acrylamide in pretzels cannot be attributed to sodium bicarbonate, as the increase in pH causes a decrease in acrylamide formation.

Another ingredient on which to focus attention is certainly salt (NaCl) which, in addition to being a flavor enhancer, is involved in the structure and formation of the gluten network, increasing the stability, flexibility and resistance of the dough, decreasing its absorption of water. Furthermore, it slows down the activity of the yeast present in the dough.

Salt has shown a notable inhibitory effect on acrylamide formation but high levels can nullify this effect. It has been shown that adding 3% NaCl, instead of 1%, to bun dough reduces the acrylamide content due to lower amylase activity and, therefore, reducing sugar content [56].

The concentration of asparagine also decreased when 3% salt was added to the dough. Conversely, higher concentrations of salts significantly increased the amount of acrylamide in the product. In the specific case, i.e. pretzels, there is a high concentration of salt which could induce the formation of acrylamide, as it is present in the dough but also on the surface. Therefore, the high acrylamide content in pretzels is undoubtedly attributable to the asparagine content present in the flour but also to the salt also present in the portion of the product which is in close contact with the heat source and, obviously, during cooking.

The absence of acrylamide in the fried pizza samples could be attributed to yeast. As the yeast induces the fermentation process by which the sugars are transformed into carbon dioxide (CO2) and ethyl alcohol. CO2 is the leavening agent while the alcohol evaporates during the cooking process.

During the fermentation process yeast can use asparagine for its metabolic activity [57]. Consequently, a prolonged fermentation time could reduce the formation of acrylamide; this is the case of fried pizza as it is characterized by a leavening of the dough equal to six hours. This could be the reason for the results obtained since fried pizza is not the only product to contain wheat flour but it is the only product with this ingredient to report values below the LOD.

In addition to fried pizza, tacos were also the second and last products to report values below the LOD. For the preparation of the tacos, cornmeal, water, oil and salt are used. Cornmeal has a low free asparagine content of 4.28 mg per 100g of flour [7]. In fact, even the scagliozzi, fried pieces of polenta typical of Neapolitan street food, which contain corn flour reported low acrylamide values and only 7 out of 20 samples, above the LOD.

Regarding the olive-based products are concerned, an ingredient often cited in scientific literature due to the high level of acrylamide in its derivative products [58], Ascoli olives were analysed. These have undergone an exception regarding the method of extraction; during the weighing for the extraction, in addition to the external rind, part of the olive was also taken. This is because the FDA (Food and Drug Administration) in 2003 reported for the first time the presence of acrylamide in canned black olives with concentrations of about 2000 µg/kg [59]. While in green olives they reported maximum concentrations of 233 µg/kg [60].

The enormous difference in concentration found in black and green olives can be explained by the influence of the different processing methods in order to make the product edible. Initially there is a debittering process with the aim of hydrolysing and making oleuropein soluble, the compound responsible for the bitter taste present in olives. Oleuropein is split into glucose, elenolic acid and hydroxytyrosol, metabolites which are subsequently removed by washing with water. In the de-bittering phase, soda is used in a concentration of between 1.50% and 3%, a concentration that varies in relation to the ambient temperature, the variety of olives and the state of ripeness of the drupes. After the debittering process, it follows washing, pickling, fermentation and packaging.

The fermentation can be of different types, among the main ones, the Spanish, Greek and Californian methods. Acrylamide appears to be formed only in the processing of ripe black and green Californian-type olives [59].

In this process, the olive undergoes to a series of treatments with solutions diluted in sodium hydroxide (NaOH) and there is a chemical debittering. Three treatments are usually carried out: the first is defined as superficial (epidermal), the second allows the alkaline solution to penetrate the pulp up to half of its thickness, with the third the alkali reaches the stone of the olive. The immersion in sodium solutions is repeated at least three times and, between two successive treatments, the olives are washed in water and subjected to oxidation by blowing in air under pressure. The air oxidizes the polyphenols which cause the blackening of the fruit. In order not to obtain color variations of the olives during storage, the product is stabilized with the addition of ferrous salts (ferrous gluconate and ferrous lactate). At this point the olives are washed again with water, placed in brine and sterilized, given their high pH [61].

Peptides that bind to oxidized polyphenols and other carbonyl donors such as elenolic acid monoaldehyde act as precursors for acrylamide formation in olives.

Green olives recorded a lower acrylamide content because the Californian style excludes storage in brine and the oxidation phase in air. In fact, it was evaluated that olives processed without air oxidation had significantly lower acrylamide levels [62]. This may explain the reduced formation of acrylamide in the case of stuffed olives, which are green olives. Unfortunately, we were unable to trace the type of processing used.

Also, the sterilization phase seems to be responsible for the presence of acrylamide, in fact in pasteurized olives the acrylamide content is lower [59].

For the preparation of Ascoli olives, the drupe after pitting is filled with a mixture of meat cooked with white wine, salt, onion and carrot. It is then covered in flour, egg and breadcrumbs and is ready to be subjected to the final frying.

During the frying process, the inside of the olive remains soft because most of the water reaches boiling temperature. The outer crust confines the moisture inside the product and this causes the inside to be cooked.

In addition to the fermentation process, cooking can also lead to a reduction in the amount of phenolic compounds in the olive, with a greater increase in acrylamide [62]. However, the frying of the stuffed olives generated a reduced concentration of acrylamide because, being a short-term cooking, the quantity of phenolic compound is preserved, thus decreasing the formation of acrylamide, in addition to the fact that probably the olives used were not subjected to insufflation of the air and sterilization.

Another parameter to be considered is the addition of antioxidant compounds to the dough before the cooking process, a procedure used to reduce the formation of acrylamide, as it inhibits the formation and speed of the Maillard reaction. The positive effect is attributable to the trapping capacity of the carbonyl compounds. For example, rosemary may reduce acrylamide formation in bread [63]. However, this positive effect is not reported for all antioxidants as conflicting opinions have been found.

For the production of falafel, cumin was used, a spice that has an antioxidant action. The percentage of samples analysed having detectable acrylamide was 100%, with a maximum value of 73.5 µg/kg, a minimum value of 45.5 µg/kg and an average of 55.55 µg/kg.

The functional group in position α and γ of the carbonyl group could be the key factor of the interaction with asparagine for the formation of acrylamide, as well as the α, β, γ, δ-diunsaturated carbonyl groups [63]. Then antioxidants bearing such functional groups can react with asparagine to produce acrylamide.

The reduced concentration of acrylamide in falafel could be attributable to the cumin but also to the chickpeas present, as products containing chickpeas or chickpea flour have reported very low acrylamide concentration values. Hence, it is assumed to be related to chickpeas rather than cumin. The figure 21 represents the correlation between the different types of cooking and the formation of acrylamide. Therefore, it was found that the type of cooking influences the production of acrylamide. However, there was a higher incidence of positive samples in baked products (96.3%), followed by pan-fried products (73%) and fried products (68.2%).

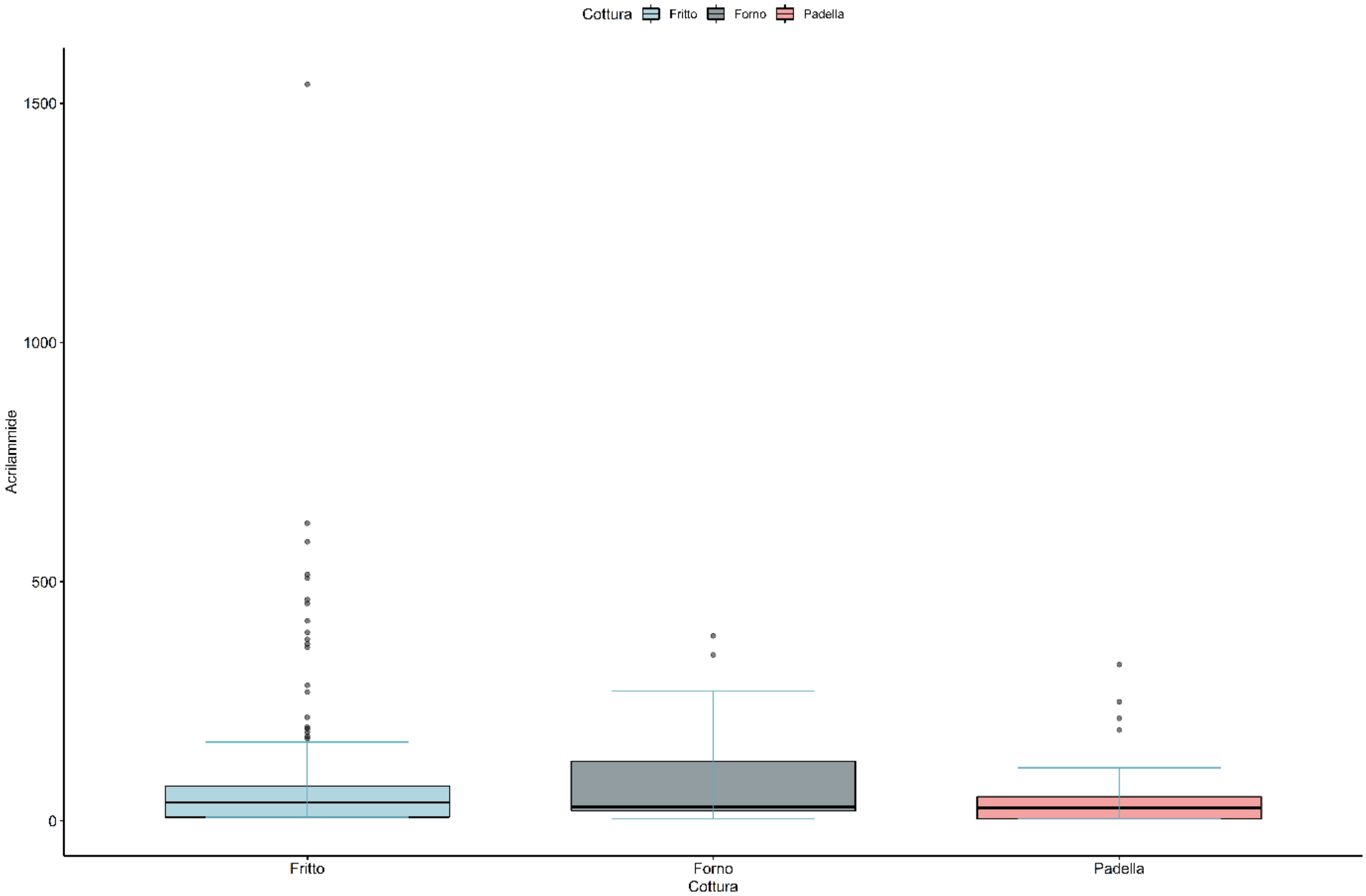


Figure 21. Box-whisker plot of the AA concentrations sorted by cooking type. Fritto = fried; Forno = oven; Padella = cooking pan.

The baked samples revealed the highest acrylamide mean contents (77.67 ± 83.01 µg/kg), followed by the fried products (67.65 ± 129.12 µg/kg) and pan cooked (38.20 ± 49.24 µg/kg). It can be seen that fried and pan-fried products have a greater scatter than oven-baked products. This is because the temperature in pan-fried and/or fried products is less controllable than in the oven and therefore they are more prone to greater variability for the production of acrylamide.

In general, cooking is a process that consists of exposing a product to heat to transform it from raw to cooked.

In baking, heat is applied directly to the food product, usually in an oven reaching temperatures up to 260°C [55]. High temperatures and low humidity levels are responsible for a series of chemical reactions between food components such as the formation of acrylamide.

The thickness of the product also determines the drying rate and consequently the rate of acrylamide formation during firing [55]. The street food products that need to be cooked in the oven were fügassa, bretzel, cecina and erbazzone.

Frying is a cooking technique which consists in introducing a food into a fatty medium at a high temperature (160-200°C), where the oil or fat acts as a heat transmitter producing rapid heating which results in a golden and crunchy food [62]. The heat propagates by convection, i.e. it reaches the cold food through the liquid used. Frying showed the higher dispersion of data because the product is immersed at a lower temperature than the oil, resulting in a lowering of the temperature or continuous variations in temperature; for this reason there are different concentrations of acrylamide in the final product.

The fried products analysed were arancini, fried pizza, panissa, sgabei, olive all'ascolana, fried cream, scagliozzi, panzerotti, panelle, croquettes, seadas, falafel, cannoli and courgette flowers.

Regarding the pan cooking, the heat propagates directly but, even in this case, there are temperature variations which determine different concentrations of acrylamide. The products cooked in a pan were piadina, crepes, dosas, tacos and tortillas.

In addition to the cooking method, time must be taken into consideration, as frying and pan cooking require less cooking times than oven cooking [64]. Baking maintains a constant temperature for an extended period, which is ideal for the formation of acrylamide.

Figure 22. Box-whisker plot of the AA concentrations obtained for the products cooked in oven. Black bar corresponds to the median.

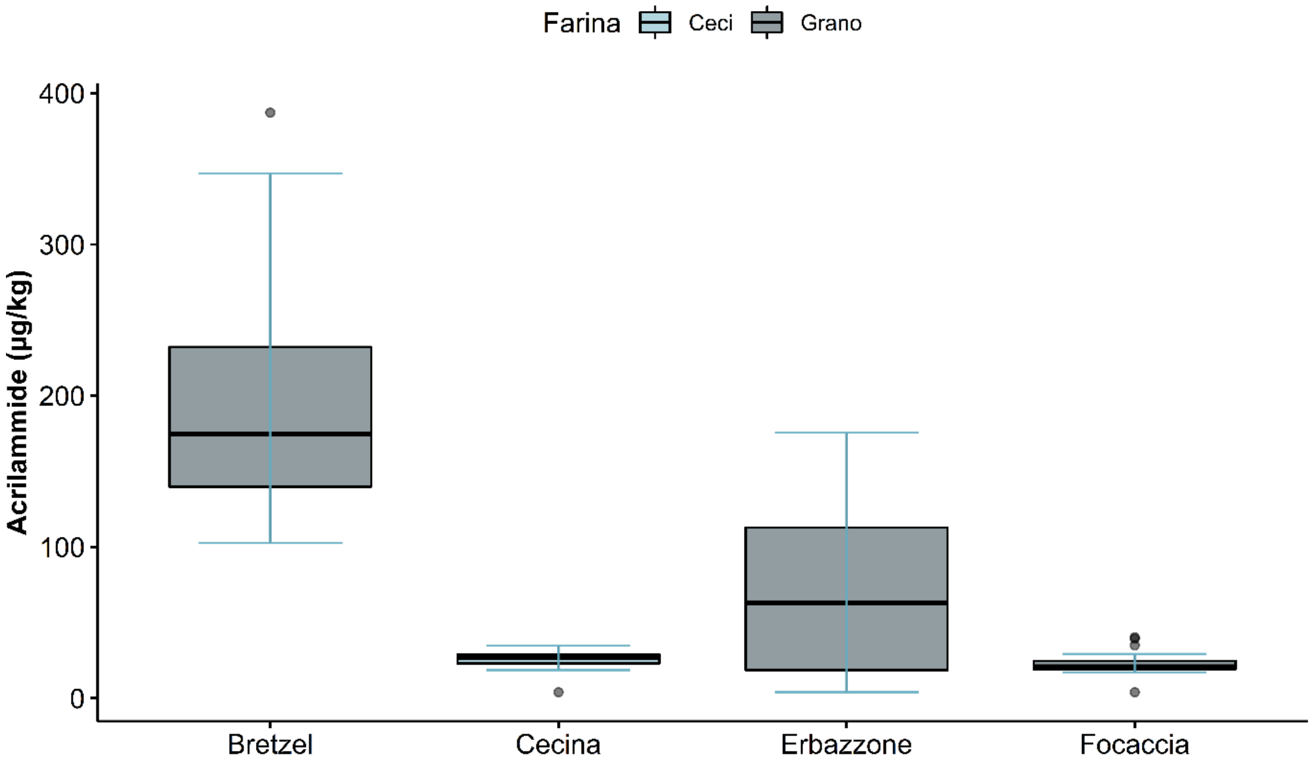


Figure 22 shows the AA levels found in bakery products. The parameters that influence the formation of acrylamide in bakery products can be technological and compositional.

The parameters linked to the composition of the product refer to the presence of asparagine, sugars and yeast or chemical leavening agents but also salt, water and additives (antioxidants, acids, enzymes, cations, amino acids) [55]. While the technological parameters concern time and temperature, pH, physical parameters, fermentation time and humidity.

Bretzel and erbazzone are characterized by higher acrylamide values, whereas focaccia and cecina showed lower AA values.

What the bretzel and herbaceous have in common is certainly wheat flour which has high values of free asparagine [65]. However, the focaccia is prepared with wheat flour but undergoes to a leavening process which could be the reason for the determination of slightly lower acrylamide values compared to bretzels and erbazzone. On the other hand, cecina showed very low acrylamide values as chickpea flour is present and all the products having this primary ingredient showed low acrylamide levels, regardless of the type of cooking.

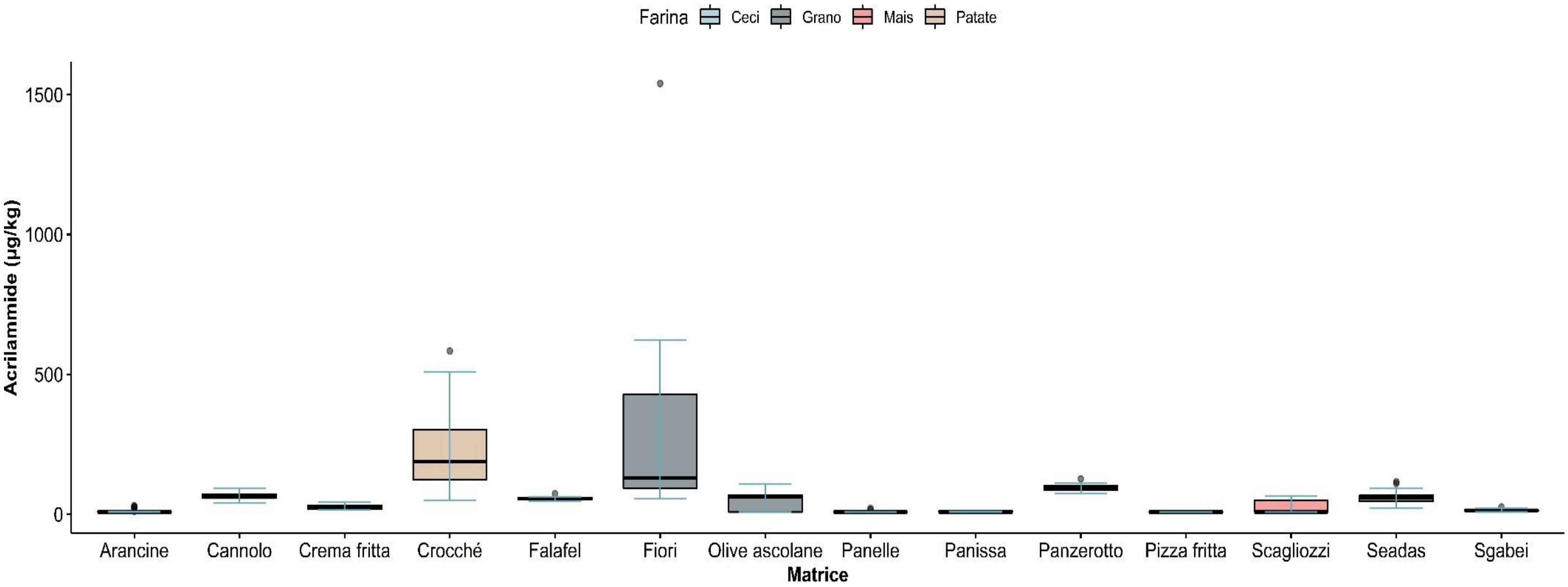


Figure 23. Box-whisker plot of the AA contents of fried products sorted by sample type. Black bar corresponds to the median.

The Figure 23 shows the concentration of acrylamide in fried products.

Courgette flowers report the highest acrylamide values, followed by croquettes, panzerotti, cannoli, seadas, falafel, stuffed olives, fried cream, scagliozzi, sgabei, arancine, panelle, panissa and fried pizza.

As for bakery products, the fried products containing wheat flour report the highest acrylamide values, followed by corn flour (scagliozzi) and chickpea flour (panelle and panissa). However, this order is not always respected and changes on the basis of the type of product. In fact, fried pizza has no detectable acrylamide values despite being based on wheat flour. In contrast, falafels showed higher average acrylamide concentration despite being chickpea-based.

The parameters that might be related to these exceptions are different. As for falafel, the higher concentration compared to other chickpea flour products could be attributed to cumin as the function of antioxidants is not yet fully understood. In fact, there are antioxidants that reduce the concentration of acrylamide (e.g. rosemary) and others that can increase it (e.g. curcumin) or have no effect. Cumin could increase the concentration of acrylamide [63].

Sgabei have low acrylamide contents as the recipe contains yeast which could use free asparagine for the metabolic activity reducing the final concentration [55]. Even for fried pizza, in which there are 6 hours of leavening, undetectable acrylamide values were found despite being a wheat-based product.

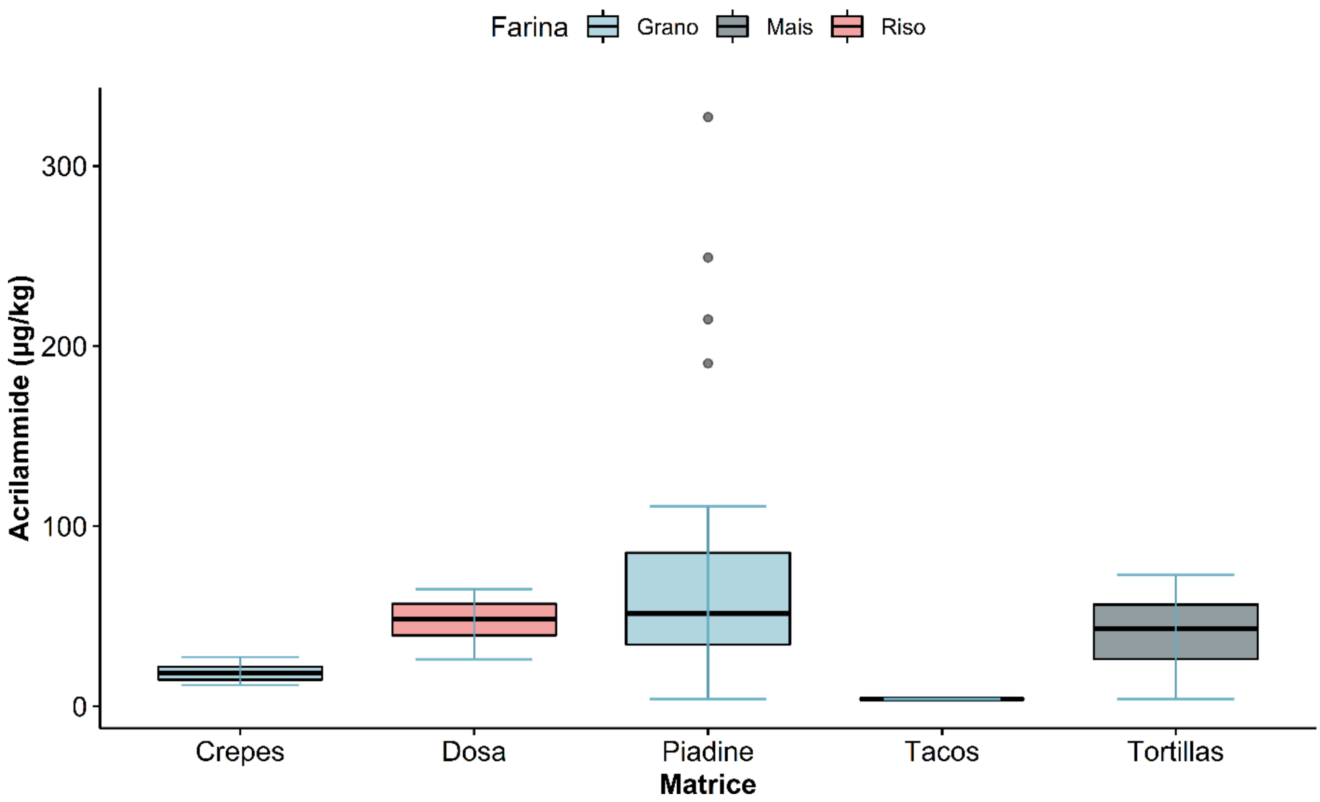


Figure 24. AA contents of street food samples cooked in pan. Black bar corresponds to the median; circled correspond to the outgroups.

Piadine, tortillas, dosas, crepes and tacos represent products cooked in a pan.

The piadine and tortillas are made from wheat flour and therefore it is expected that there are higher concentrations of acrylamide. These are followed by the dosa which consist mostly of rice. As for the crepes, they should be in the third place, but the cooking time is short enough to keep them soft and therefore, this could be the consequence of the reduced concentrations of acrylamide. Tacos, as fried pizza for fried products, showed no detectable acrylamide values; this could be due to the use of cornmeal and the reduced cooking time.

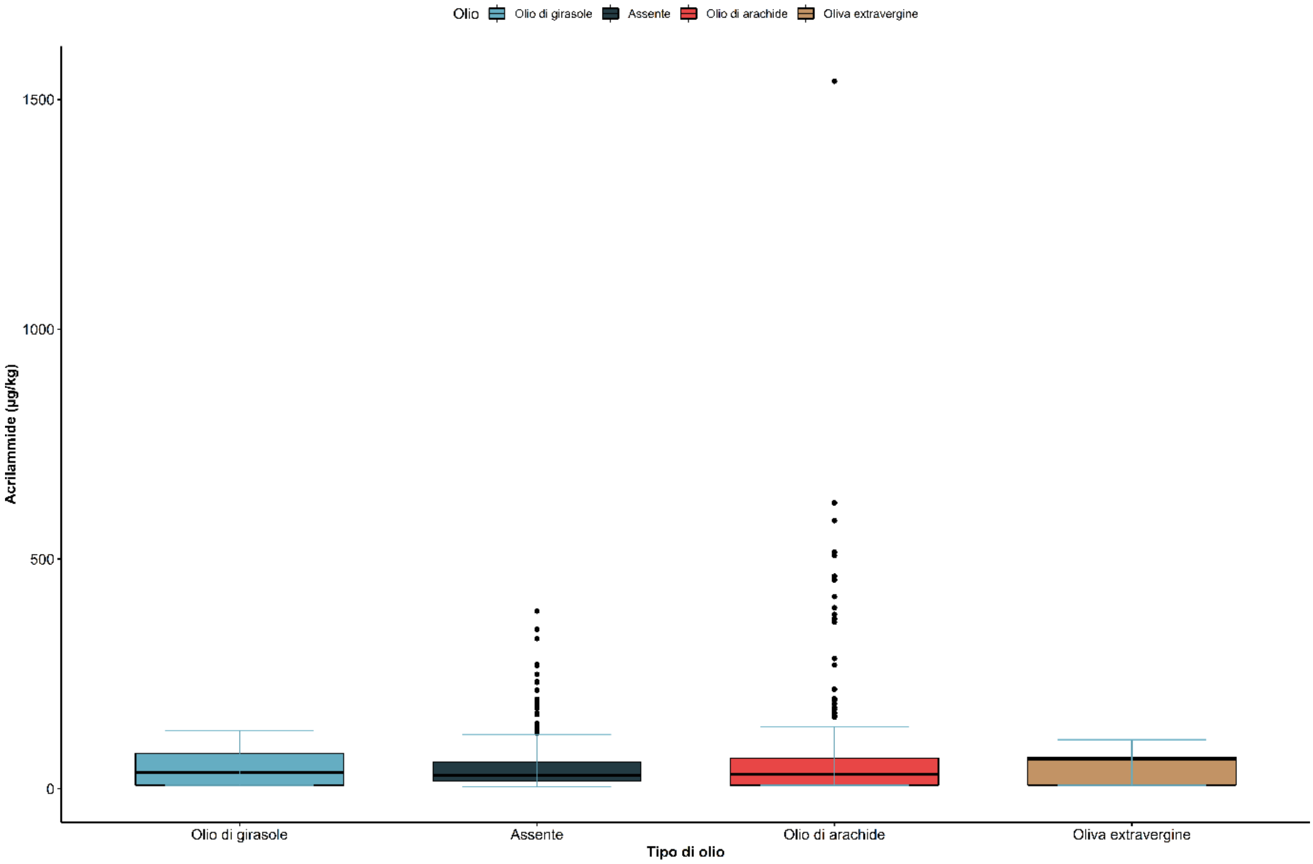


Figure 25. Acrylamide content of fried street foods sorted by types of oil used. Olio di girasole = sunflower oil; Olio di arachide = peanut oil; Oliva extravergine = extravirgin olive oil.

Figure 25 shows the concentration of acrylamide in fried street food products in relation to the different types of oils used. The oils used for frying were extra virgin olive oil, sunflower oil and peanut oil. As shown in the figure, no significant differences were found (Kruskal-Wallis chi-squared=1.4689, df=3, p-value=0.6895). This situation could be related to the chemical properties (unsaturated fatty acids, polar compounds, phenolic compounds and antioxidant capacity) of the oils but also to the frying conditions (temperature, time, difference of raw materials and equipment) [66].

The effects of different types of oils on acrylamide formation are not yet clear in the literature. The few studies present are based on french fries.

In this thesis work, no statistically significant differences were found between the various oils used despite the high number of fried products analysed (14). It can be assumed that the production of acrylamide is a function of the ingredients and the cooking process.

Furthermore, the formation of acrylamide is mainly attributable to the Maillard reaction and not to the pathway involving lipids through the formation of acrolein and the intermediate acrylic acid which, in the presence of nitrogen, can lead to the formation of acrylamide.

Street food type	Characteristic	Benchmark levels EU Reg. 2017/2158 (µg/kg)	Mean value (µg/kg)	Min-Max (µg/kg)	N. samples >LOD(%)	N. of samples above the benchmark levels (%)
Arancine	Fried Wheat flour Sunflower oil	Crunchy bread (300)	11,48 ± 7,26	8 – 30,90	25	0
Bretzel	Bakery product Wheat flour No oil used	Crunchy bread (350)	192,32 ± 76,17	102,6 – 387,10	100	1(5%)
Cannolo	Fried Wheat flour Sunflower oil	Cookies and waffles (350)	63,89 ± 13,95	39,9 - 93,20	100	0
Cecina	Bakery product Chickpea flour No oil used	Crispy bread-like products (300)	25,44 ± 6,34	3,9 – 34,90	95	0
Crema fritta	Fried Wheat flour Peanut oil	Soft bread other than wheat based bread (100)	24,75 ± 8,04	15,9 – 42	100	0
Crepes	Pan cooked Wheat flour No oil used	Soft bread other than wheat based bread (100)	18,52 ± 4,48	11,7 - 27,30	100	0

Crocchette	Fried Potato Peanut oil	Other potato paste products (750)	232,99 ± 145,10	48,9 - 583,70	100	0
Dosa	Pan cooked Rice and lentils No oil used	Crispy bread-like products (300)	46,28 ± 11,98	26 – 64,90	100	0
Erbazzone	Bakery product Wheat flour No oil used	Crunchy bread (350)	69,14 ± 50,42	3,9 – 175,50	95	0
Falafel	Fried Chickpeas Peanut oil	Soft bread other than wheat bread (100)	55,55 ± 6,62	45,5 - 73,50	100	0
Courgette flowers	Fried Wheat flour Peanut oil	Crispy bread-like products (300)	286,93 ± 345,19	55,8 - 1539,80	100	6(30%)
Fügassa	Bakery product Wheat flour No oil used	Crunchy bread (350)	22,78 ± 8,27	3,9 - 40,30	95	0
Olive ascolane	Fried Wheat flour Extra virgin olive oil	Crispy bread-like products (300)	51,32 ± 31,14	8 - 106,80	70	0

Panelle	Fried Chickpea flour Sunflower oil	Soft bread other than wheat bread (100)	9,06 ± 3,33	8 - 20,80	10	0
Panissa	Fried Chickpea flour Peanut oil	Soft bread other than wheat bread (100)	8,67 ± 1,07	8 - 11,10	35	0
Panzerotto	Fried Wheat flour Peanut oil	Crispy bread-like products (300)	94,31 ± 13,43	74,7 - 126,60	100	0
Piadine	Pan cooked Wheat flour No oil used	Soft bread other than wheat bread (100)	83,28 ± 90,02	3,9 - 327,00	100	5(25%)
Pizza fritta	Fried Wheat flour Peanut oil	Crispy bread-like products (300)	8,00 ± 0,00	8 – 8	0	0
Scagliozzi	Fried Mais flour Peanut oil	Crispy bread-like products (300)	24,13 ± 22,96	8 – 65,40	35	0
Seadas	Fried Wheat flour Peanut oil	Soft bread other than wheat bread (100)	62,04 ± 24,71	22,7 – 116,30	100	3(10%)
Sgabei	Fried Wheat flour, Peanut oil	Soft bread other than wheat bread	14,08 ± 4,55	8 – 25,80	80	0

		(100)				
Tacos	Pan cooked Mais flour No oil used	Crispy bread-like products (300)	3,90 ± 0,00	3,9 – 3,9	0	0
Tortillas	Pan cooked Wheat flour No oil used	Soft bread other than wheat based bread (100)	39,02 ± 22,58	3,9 – 72,90	100	0

Table 9. Characteristics and results of the products analysed.

4.2 Application of mitigation measures

The courgette flowers, which revealed the highest percentage of samples above the benchmark levels proposed by the EU legislation were subjected to mitigation measures in order to verified the best conditions able to reduce the AA formation.

Other courgette flowers samples were prepared at 16 different conditions, as shown on table 10.

N	F	D	O	T	MIT. CODE
1	Yes	4,5	Peanuts	162	9
2	No	4,5	Peanuts	162	10
3	Yes	4	Peanuts	162	11
4	No	4	Peanuts	162	12
5	Yes	4,5	Mais	162	1
6	No	4,5	Mais	162	2
7	Yes	4	Mais	162	3
8	No	4	Mais	162	4
9	Yes	4,5	Peanuts	153	13
10	No	4,5	Peanuts	153	14
11	Yes	4	Peanuts	153	15
12	No	4	Peanuts	153	16
13	Yes	4,5	Mais	153	5
14	No	4,5	Mais	153	6
15	Yes	4	Mais	153	7
16	No	4	Mais	153	8

Table 10. Scheme of the different conditions carried out for the mitigation study. F = freezing of the samples for 5 min; D = time of frying; T = temperature of frying; O = type of oil used.

Four factors were considered for the mitigation studies: freezing at -20°C for 5 min, Time, Temperature and type of oil used, as shown in table 11.

FACTOR	SYMBOL	-	+	UNITS	STD
FREEZER	**F**	Sì	No	°C	200
DURATION	**D**	4,5	4	Minutes	5
OIL	**O**	Peanuts	Mais	-	-
TEMPERATURE	**T**	162	153	°C	180

Table 11. Factor considered for the mitigation study. STD = standard conditions of cooking.

Ten samples of courgette flower were produced for each mitigation condition, for a total of 160 samples. The courgette flowers obtained were subjected to the organoleptic evaluation by 7 expert panelists taking into account 5 parameters color, smell, appearance, consistency and taste. Each panelist assigned a score from 1 to 7 where 1 corresponds to poor and 7 to excellent.

Figure 26 shows the box whisker plot of the acrylamide concentrations obtained sorted by different mitigation procedure. We can see a great variability between the different measures. 16. Considering an acrylamide concentration at standard condition of 286 ug/Kg, we found a significant decrease with the conditions 9, 16 and 14 (p = 0.000074).

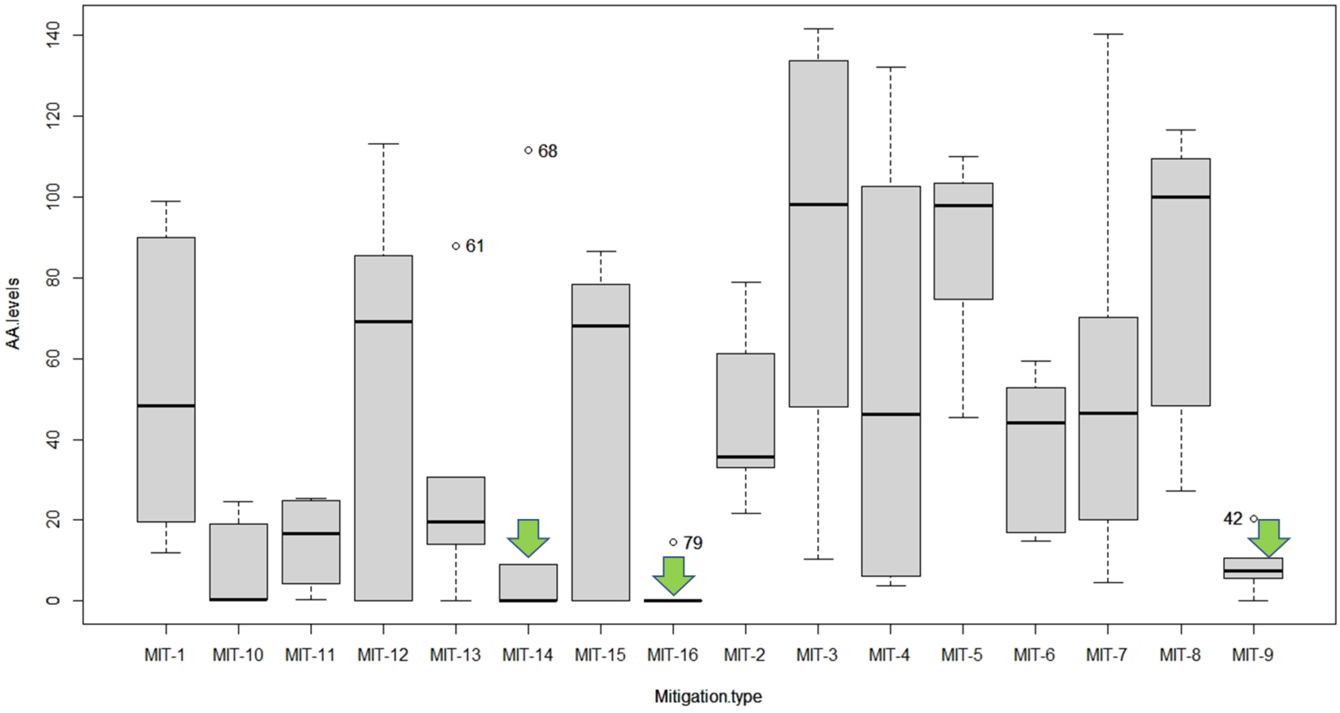


Figure 26. Box whisker-plot of the AA contents found in the courgette flowers subjected to mitigation measure.

The mitigation measure 16 revealed the lowest acrylamide concentrations. Figure 27 shows the differences between the standard conditions and the mitigations measures of the samples analysed.

Figure 26. Color analysis of the courgette flower prepared at standard conditions and at different mitigation measures.

5. CONCLUSIONS

The pilot study on the samples of street food produced and marketed in Italy verified a high heterogeneity in the concentration of acrylamide. The latter depends on the ingredients, the cooking technique and the preparation of the product.

The high acrylamide values found in courgette flowers, brezels and croquettes suggest that mitigating measures should be taken. These could include variations in the cooking time and temperature maintaining intact the organoleptic properties of the products.

The most common technological approaches regarding the possible mitigation of acrylamide are related to changes in raw materials (e.g. selection of plant varieties with low content of precursors), in the recipe or formulation, in the use of pre-treatments (such as blanching or) and changes in processing conditions (significant change of time, temperature, pressure and pH of heat treatment) [67]. Cations also seem to reduce the formation of acrylamide and the greatest efficacy was found in the bivalent cations (Ca^{2+} and Mg^{2+}) compared to the monovalent ones (Na^{+} and K^{+}). Divalent cations resulted in an approximately 30% decrease in crackers and breads [55].

Chlorine chloride (CaCl2) is the most effective inhibitor of acrylamide formation even compared to calcium carbonate (CaCO3). Furthermore, the addition of metal cations to certain foods can cause a decrease in pH and induce a reduction in acrylamide content [68].

For courgette flowers, the mitigating measures that could be applied concern the use of different types of flour (wheat, rice, corn, soy, chickpeas) and the addition of other ingredients (water, milk and eggs). For example, it seems that the use of egg together with flour, instead of water or milk, reduces the amount of acrylamide present, regardless of the type of flour used [49]. However, this could compromise the organoleptic properties of the product.

For olives, changes could be made in the flow sheet such as washing, storage time in saline solution, decrease in oxygen exposure time, increase in pH before sterilization, reduction in sterilization temperature and time, use of additives.

In general, for potato products, you could soak the potato slices in water or a citric acid solution. This can reduce acrylamide levels in chips by up to 40 or 75%, respectively [17]. This consideration can be extended to all types of potato products.

During this study we had the opportunity to understand that the cooking process influences the formation of acrylamide. The conventional cooking process is mainly divided into two phases: development phase and drying and coloring phase.

In the development phase, the temperature of the dough starts to increase causing some physical changes in the dough (e.g. starch gelatinization). Subsequently, both the humidity and temperature conditions become favorable for the browning reactions, which occur in the last phase and which also involve the formation of acrylamide. Precisely for this reason, one of the mitigating measures could be to minimize the effect of the thermal process on the formation of acrylamide. Therefore, vacuum technology has recently been included to decrease the process temperature and therefore also the formation [69].

Vacuum technology is based on the accelerated drying process at lower temperatures, as the sub-atmospheric pressure lowers the boiling point of water and leads to evaporation at lower temperatures and times. In this way, drying takes place without exposing the product to high temperatures, unlike what occurs with conventional atmospheric drying [70]. Furthermore, the vacuum has an increasing effect on the internal vapor pressure of the material to be dried, leading to increased moisture migration.

Vacuum technology has been introduced in the biscuit baking process [71]. These were subjected to convection cooking (180, 190 or 200 °C) or vacuum cooking at 500 mbar (160, 170 or 180 °C) for different times. Conventional cooking, compared to vacuum cooking, revealed higher amounts of acrylamide in the biscuits. The study also revealed that low pressure applied during sous vide cooking accelerated the drying rate of the biscuits [71].

In addition to sous-vide cooking, vacuum frying could also be used, which was first introduced for deep-fried fruit and vegetable products [72].

Sous-vide frying is a cooking process operated below atmospheric pressure, generally <6.65 kPa [73]. This lead to a reduction of the boiling point of oil and water, which improves quality attributes of the fried product, such as oil content, texture, nutrient retention and color, as well as resulting in a reduction of acrylamide formation. In fact, it has been demonstrated that the use of this technology in chips has led to a reduction in the temperature of the frying process and a consequent reduction of the formation of acrylamide equal to 94% [74].

In addition to vacuum heating also other unconventional treatments such as microwave heating, irradiation, ultrasound, high hydrostatic pressure (HHP), air jet impact, pulsed electric fields (PEF), supercritical fluid technology (SFT), radio frequency (RF), extrusion, infrared (IR) radiation and steaming could be used for the reduction of acrylamide in different food products [67]. For example, PEF, irradiation, SFT and HHP treatments are considered non-thermal processes that support the removal of asparagine and reducing sugars from raw materials, resulting in the inhibition of acrylamide formation [75].

In conclusion, acrylamide is a substance that is formed following the high-temperature cooking of foods rich in reducing sugars and asparagine and is classified as a probable human carcinogen. The types of street food products analysed revealed more or less high levels of acrylamide, with higher concentrations in courgette flowers, croquettes and pretzels. However, it is assumed that the intake of these foods is sporadic and therefore their overall contribution to dietary exposure is limited, especially if a balanced diet is followed. Finally, the results from the application of mitigation measures revealed the important role of this practice to prevent the risk of acrylamide formation, preserving the quality of the product.

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