Mitigation strategies to reduce food-processing contaminants formation in Neapolitan pizza

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This PhD thesis aimed to evaluate possible mitigation strategies to contain food-processing contaminants formation during Neapolitan pizza production. In particular, the obtainment of a low-asparagine flour for the production of Neapolitan pizza with lower potential to generate acrylamide during baking has been evaluated. Moreover, the effect of different baking process (wood-fired oven and electric oven) on the formation of contaminants such as acrylamide and polycyclic aromatic hydrocarbons was assessed.

Strategie di mitigazione per la riduzione della formazione di contaminanti di processo nella pizza Napoletana

# Questa tesi di dottorato ha riguardato la valutazione di possibili strategie di mitigazione della formazione di contaminanti di processo durante la produzione di pizza Napoletana. In particolare, è stata valutata la possibilità di produrre sfarinati con minore contenuto in Asparagina, e quindi con più basso potenziale di formazione di acrilammide durante la cottura di pizza Napoletana, e l’effetto di modifiche del processo produttivo (cottura in forno elettrico e in forno a legna) sulla formazione di contaminanti quali acrilammide ed idrocarburi policiclici aromatici.

# **Key words**: Acrylamide; polycyclic aromatic hydrocarbons (PAHs); Neapolitan pizza; mitigation strategies.

# **1. Introduction**

Following the previous annual report of this PhD thesis project (Quiquero, 2022), the present oral communication illustrates the main results of the following activities:

A) utilization of wheat varieties with low Asparagine (Asn) content for Neapolitan pizza production;

B) production of Neapolitan pizza from low Asn wheat line according to Reg. EU 2017/2158;

C) effect of different baking process (wood-fired oven and electric oven) on the contaminants’ formation.

**2. Thermal food processing - background**

Thermal processes such as frying, baking, and roasting are commonly applied to foods for processing or preservation purposes. They provide the final product with specific flavor, aroma and texture characteristics but toxic compounds can be formed due to the high temperature reached during process, with a deleterious effect on the food quality and safety. The presence of varying amounts of thermal process contaminants in widely consumed foods daily is considered as a major concern by public authorities worldwide; in bakery products the major issue is related not to the level found in food but to the high consumption that make these products a potential risk for human exposure. Therefore, efforts to reduce the amount of these contaminants in heat-treated foods have gained importance (Hamzalioglu 2020; Nerìn et al., 2016). Neapolitan Pizza is one of the most appreciated and popular Italian foods in the world and it has acquired a global significance during last years; in 2010 it has been recognized as a “Traditional specialty guaranteed” (TSG), thanks to EC Regulation 97/2010, and in 2017 received recognition by UNESCO as an “Intangible Cultural Heritage of Humanity”. In compliance with the cooking standards provided by the disciplinary of production, Neapolitan pizza is cooked in wood fired ovens with temperature of the dome and bed equal to 430°C and 485°C, respectively, and with a cooking time not exceeding ninety seconds (Falciano et al., 2022). In these conditions, a unique flavor and aroma was provided to pizza, but neo-formed contaminants can be produced. In particular, this study focuses on two food-processing contaminants that can be formed during Neapolitan pizza cooking, acrylamide and polycyclic aromatic hydrocarbons. Acrylamide has been classified as a “probable human carcinogen” (group 2A) with genotoxic and carcinogenic effects. This compound generates during the Maillard reaction from Asn and reducing sugars, naturally present in carbohydrate-rich foods. In 2017, the Commission Regulation 2158 established mitigation measures and benchmark levels to reduce acrylamide content in various food categories; European food safety authority highlighted mean acrylamide concentration in bakery products between 40 and 231 µg/kg, and indicated that ALARA principle should be applied during processing to reduce its levels in the final product, hence reducing consumers exposure. To this aim, food industry and national authorities of the European Union cooperate for the FoodDrinkEurope Acrylamide “Toolbox” as a tool to implement this regulation through improvement strategies during manufacturing processes.

In this research project different crops management strategies were used to produce grains with different Asn content in order to exploit agronomic practices as a potential strategy to mitigate acrylamide content in the final products. The rationale of our experimental approach is that in cereal grain free Asn content has been identified as the main determinant of acrylamide-formation during processing (Raffan 2019). Polycylclic aromatic hydrocarbons (PAHs) are formed and released via the pyrolysis or incomplete combustion of organic materials, and during the industrial processes such as smoking, frying, drying, baking, roasting and charcoal barbecuing (Kaknaz et al., 2019), and can impact both nutritional value and air quality. Research to reduce PAHs contamination and assure food safety and quality in the bakery chain included type of cooking method used, which can have a pronounce impact on PAHs contamination (Singh et al., 2016). To date, no data are available concerning PAHs formation in Neapolitan pizza. In this research project we first assessed the formation of PAHs during traditional cooking of Neapolitan pizza in wood-fired oven; subsequently, the potential formation PHAs was determined using electric oven to assess differences with the traditional approach.

# **3. Materials and Methods**

# **3.1 Sample obtainment**

# *Wheat samples*

# Soft wheat of LG Ayrton variety was provided by the University of Torino. Samples consisted of 10 different wheat lots (T2, T6, T7, T11, T12, T13, T15, T17, T19, T20) grown in experimental fields in Piemonte (Italy) during the season 2021/2022. Crop management of different fields was performed by testing different nitrogen dose, sulfur supply, and fertilizer type. Wheat samples have been analyzed for grain characteristics as test weight (kg/hL), performed using a Shopper chondrometer and thousand-kernel weight, TKW (g). Grain samples were milled with a laboratory mill and tested for chemical traits including moisture (ICC method 104/1), protein (AOAC method 992.23), ash (ICC method 104/1) and free Asn content.

# *Grain milling and flour selection*

# Aliquots (20kg) of wheat lots were reunited on the basis of their Asn content, then tempered (moisture of 16%) for 16 h to allow an adequate distribution on the surface and milled with a soft-wheat mill (NAMAD SG2000, Rome, Italy), equipped with three break, three reduction rolls and six steel screens, obtaining three breaking rolls flours (B1, B2, B3), three sizing rolls fractions (C1, C2, C3), and bran and shorts. Milling fractions were analyzed for moisture, protein, ash and Asn content. Milling fractions were recombined taking into account Asn content, milling yield, protein, and ash content. Three flours with different Asn content have been obtained: a “00” type flour (F00) with low Asn content by recombining B1, B2, C1, C2 and C3 fractions of the mix low asparagine content (T2, T6, T7, T11), a “0” type flour (F0) by recombining B1, B2, B3, C1, C2 and C3 fractions and a whole-wheat flour (FI) with C1, C2, C3, bran and shorts fractions, from varieties with high asparagine content T15, T19 and T20.

# *Dough’s preparations and cooking conditions*

# Dough’s preparation and samples obtainment were set up according to the traditional manufacturing process of Neapolitan pizza (Commission Regulation EU 97/2010). Pizza doughs were prepared by mixing 1200 mL of water, 2300 g of the selected “00”, “0” and “whole-wheat” flour and 2% of sodium chloride. Two different pizza typologies have been obtained, with a topping of tomato sauce and without any topping, both cooked in wood fired oven and in electric oven at 485°C for 90s. Prior analysis, Neapolitan pizza samples were lyophilized using a Virtis Genesis 25ES freeze drying apparatus.

# **3.2 Chromatographic analysis**

# *Sample extraction and chromatographic conditions for Asn analysis*

# Asn determination was carried out on soft wheat varieties. The method used for aminoacids extraction was the same illustrated by Curtis et al., 2009, with slight modifications. To 0.5g of finely ground whole-grain flour, 10 ml of 0.01M HCl was added; the sample was stirred for 15 minutes at room temperature and then allowed to stand for a further 15 minutes. An aliquot (1.5 ml) of supernatant was centrifuged at 5000 rpm for 15 minutes. Aliquots of 20 µL of sample were injected into HPAEC-PAD for free Asn analysis after appropriate dilution.

# Separation and quantification of free Asn was conducted using an HPAEC-PAD system, model ICS 6000 (Thermoscientific, Milan, Italy). An AminoPac PA-10 column (2x250mm) was employed for chromatographic separation using as eluents H2O (eluent A), NaOH 250mM (eluent B), acetate 1M (eluent C) with a flow of 0.250 mL/min in a gradient composed as follows: 0-12 min 80%A and 20%B, 12-16 min 68%A and 32%B, 16-40 min 36%A, 24%B and 40%C, 40.1-42.1 min 20%A and 80%B and 42.2-62 min 80%A and 20%B. Using these conditions, asparagine-related peak appeared at 6 minutes and chromatograms were processed using Chromeleon version 7.2.10 (Thermoscientific, Milan, Italy).

# *Sample extraction for PAHs analysis and chromatographic conditions for PAHs analysis*

# For PAHs extraction, lyophilized samples were weighted and added with an appropriate quantity of water to reconstitute the sample with the initial moisture value and obtain 5g of wet sample. Acetonitrile-based extraction was performed in a 50mL centrifuge tube mixing the sample with 1 ng/g of spiking standard (PAH calibration mix) and using Tryphenylene (10 ng/mL) as internal standard. After the addition of 5 mL of deionized water and 5 mL of acetonitrile, samples were vigorously vortexed and left overnight. Afterwards, 2.5 g anhydrous magnesium sulfate and 2.5 g sodium chloride were added, followed by shaking and centrifugation at 4000 rpm for 5 min. Then, 3 mL of the upper layer acetonitrile extract were evaporated to dryness under nitrogen stream at room temperature. The residue was dissolved in 100 μL of acetonitrile and injected into the HPLC-FLD.

# Gradient elution and fluorescence detection excitation/emission program were set up according to the method described by Viegas et al. 2012. The mobile phase was as follows: solvent A: 75% methanol (in water); solvent B: 100% methanol, solvent C: 100% ethyl acetate with a flow rate 1ml/min. The linear gradient program was: 0–18min, 0–80% B in A; 18–19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28.5 min, 90–82% B in C; 28.5–37.5 min, 82–80% B in C; 37.5–40min, 80–100% B in C, 40–45min 100–0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected were 276/330 nm for Na (naphthalene), Ac (acenaphtene) and F (fluorene); 250/336nm for Pa (phenanthrene); 250/402nm for A (anthracene); 270/460nm for Fl (fluoranthene); 270/390 nm for P (pyrene), BaA (benzo[a]anthracene) and Ch (chrysene); 260/430 nm for BbF (benzo[b]fluoranthene); 290/410 nm for BkF (benzo[k]fluoranthene), BaP (benzo[a]pyrene), DhA (dibenzo[a,h]anthracene), and BgP (benzo[g,h,i]perylene); 290/470 nm for IP (indeno[1,2,3-cd]pyrene).

# *Statistical analysis*

# Statistical analysis was performed with IBM SPSS Statistic Base (Version 29). Data were subjected to analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Results with p < 0.05 indicate statistically significant difference. Pearson correlation coefficients were calculated using IBM SPSS Statistic Base (Version 29).

# **4. Results and Discussion**

## **4.1 Obtainment of flour low in Asn by acting on agronomic practices**

## Table 1 reports the results concerning chemical composition and Asn content of wheat lines obtained using different cropping practices.

## **Table 1** *Chemical composition and Asn values of wheat lines obtained from different field management practices.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **TKW****(g)** | **Test weight (kg/hL)** | **Moisture****(%)** | **Ash****(%d.w.)** | **Protein\* (%d.w.)** | **Asn****(g/kg d.w.)** |
| **T2** | 37,8±0,6abc | 83,5±0,0bc | 11,5±0,13cd | 1,77±0,024ab | 11,1±0,02g | 0,371±0,006ed |
| **T6** | 36,8±0,9bc | 82,9±0,0d | 11,6±0,20bcd | 1,75±0,008bc | 11,2±0,06g | 0,374±0,006ed |
| **T7** | 39,4±0,9a | 83,8±0,0cd | 11,7±0,06bcd | 1,72±0,008cd | 12,2±0,03e | 0,355±0,008e |
| **T11** | 37,5±0,5abc | 84,0±0,0a | 11,4±0,18d | 1,66±0,008d | 12,7±0,14d | 0,353±0,026e |
| **T12** | 39,1±0,6ab | 83,0±0,5d | 11,5±0,10cd | 1,71±0,024cd | 11,8±0,14f | 0,405±0,021ed |
| **T13** | 38,2±1,1abc | 83,2±0,1cd | 12,3±0,10a | 1,78±0,008ad | 12,4±0,02e | 0,392±0,022ed |
| **T17** | 39,4±0,9a | 83,1±0,0cd | 11,7±0,15bcd | 1,81±0,000a | 12,3±0,06e | 0,427±0,024cd |
| **T15** | 38,3±0,9abc | 83,3±0,0cd | 11,7±0,04bcd | 1,74±0,020bc | 14,6±0,04a | 0,493±0,006ab |
| **T19** | 36±1,0c | 83,3 ±0,0cd | 11,9±0,07b | 1,79±0,016ab | 13,6±0,11c | 0,470±0,030bc |
| **T20** | 38,5±1,1ab | 84,0±0,0a | 11,8±0,15cb | 1,75±0,024bc | 14,1±0,20b | 0,543±0,042a |

*Mean values ± sd. Different letters in a column indicate statistically significant differences (p<0.05, T – test). TKW, thousand kernel weight; \*N x 5.70;*

The results highlighted that the Asn content of different wheat lines analyzed ranged between 0.353 to 0.543 g/kg d.w., indicating that different agronomic practices are a promising approach to obtain a variable Asn composition of wheat lines (Fig. 1a).

c

a

b

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**Figure 1.** *a)**Asn content (g/kg d.w.) of wheat varieties grown using different agronomic conditions; b) Different groups with similar Asn content (g/kg d.w.); c) Correlation between Asn and protein content (% d.w.) of the tested wheat varieties; analysis have been performed through Ordinary one-way ANOVA (Tukey’s multiple comparisons test) [\*\*p<0.01; \*\*\*p<0.001];*

In particular, a reduction of 54% in Asn is achievable just by choosing the appropriate wheat variety and cropping practices. Although the results demonstrated that it is possible to obtain a variability in Asn content by acting in the field, environmental factors also have to be considered. Previous studies reported that Asn accumulation is highly influenced by environmental factors such as abiotic and biotic stresses and climatic conditions (Malunga et al., 2019; Lecart et al., 2018; Moustafa et al., 2017). Moreover, results indicates that the average Asn content of lines cultivated with the same apport of sulphur and nitrogen were remarkably similar, resulting in three different groups of wheat varieties according to their free Asn levels (p < 0.05), low, intermediate and high Asn content (Fig 1b). In particular, the group with the lowest level of free Asn (mean value 0.363 ± 0.010 g/kg d.w.) included T2, T6, T7, T11; T15, T17 and T19 had the highest content of Asn (mean value 0.502 ± 0.037 g/kg d.w.), while the intermediate group included T12, T13 and T17, with an average value of 0.408 ± 0,018 g/kg d.w.

As it concerns the chemical composition, no correlation has been found between Asn content and the other investigated traits, with the exception of a positive correlation (*r* = 0.78, *P* < 0.001) with the protein content, whose values ranged from 11.1 to 14.6% d.w. (Fig. 1c). This result is consistent with previous findings of Lecart (2018), which reported that a strong correlation between Asn and proteins was evident, especially when the protein content of the wheat flour was above 13%. Subsequently, the classes with low and high Asn content were reunited according to their Asn content and milled; in Table 2 chemical composition and Asn content of the obtained milling fraction were reported.

**Table 2**. *Chemical characteristics of milling fractions obtained from varieties with low and high Asn content.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Moisture (%)** | **Ash (%d.w.)** | **Protein\*\* (%d.w.)** | **Asn (g/kg d.w.)** |
| **low** | **high** | **low** | **high** | **low** | **high** | **low** | **high** |
| **Mix\*** | **14,0±0,06** | **14,0±0,04** | **1,79±0,03** | **1,79±0,01** | **11,0±0,07b** | **13,7±0,00a** | **0,389±0,110b** | **0,575±0,103a** |
| **B1** | 15,5±0,08 | 15,0±0,05 | 0,64±0,00b | 0,68±0,01a | 9,3±0,1b | 13,9±0,0a | 0,105±0,004b | 0,167±0,001a |
| **B2** | 15,6±0,03a | 14,4±0,12b | 0,72±0,01b | 0,75±0,01a | 11,0±0,1b | 15,5±0,1a | 0,268±0,040b | 0,273±0,010a |
| **B3** | 14,6±0,04a | 13,1±0,00b | 0,91±0,01b | 1,01±0,01a | 12,1±0,1b | 16,3±0,1a | 0,226±0,002b | 0,388±0,012a |
| **C1** | 15,6±0,07a | 14,7±0,07b | 0,56±0,01a | 0,52±0,01b | 9,4±0,0b | 11,8±0,1a | 0,113±0,003b | 0,191±0,012a |
| **C2** | 15,4±0,10a | 14,3±0,07b | 0,63±0,01a | 0,55±0,01b | 9,7±0,0b | 11,8±0,0a | 0,147±0,008 | 0,178±0,053 |
| **C3** | 15,3±0,12a | 14,3±0,09b | 0,60±0,02a | 0,55±0,01b | 9,9±0,1b | 11,9±0,0a | 0,155±0,009b | 0,206±0,010a |
| **Bran** | 14,9±0,14a | 13,6±0,03b | 5,51±0,01b | 6,04±0,02a | 14,6±0,0b | 17,5±0,2a | 1,145±0,027a | 1,062±0,041b |
| **Short** | 14,6±0,11a | 13,8±0,01b | 3,68±0,01a | 3,29±0,02b | 14,0±0,0b | 15,7±0,1a | 1,122±0,037a | 0,813±0,089b |

*Mean values ± sd. Different letters in a raw (for each parameter) indicate statistically significant differences (p<0.05, T – test). \*Obtained with T2, T6, T7 and T11 for low Asn milling and T15, T19 and T20 for high Asn milling; \*\*N x 5.70;*

As showed in figure 2b, the results highlighted a positive correlation between Asn and ash content, indicating a higher content of the Asn in the external parts of kernel. As a consequence, the more a flour is refined, the lower is its Asn content. Three different flours with different acrylamide-forming potential have been obtained through appropriate recombination of milling flours: a “00” type flour, a “0” type flour and a whole wheat flour with Asn content of 0.132, 0.202 g/kg d.w. from the milling of low Asn content group, and 0.363 g/kg d.w. from milling fractions obtained from kernel with high Asn content.



b

a

**Figure 2.** a*) Milling fraction obtained from milling of group with low and high Asn content (g/kg d.w.); b) Correlation between Asn and ash (%d.w.) of the milling fractions obtained from varieties with low and high Asn content.*

Results showed that it is possible to obtain flour with lower content of acrylamide precursor acting on agronomic conditions; moreover, through appropriate recombination of different milling flour, it is possible to realize flours with low Asn content even starting from a kernel with high Asn content.

**4.2 Evaluation of different processing conditions on food contaminants formation in Neapolitan pizza**

Flours obtained as reported in paragraph 4.1 were used as a raw material to produce Neapolitan pizza; evaluation of acrylamide and PAHs content of pizza samples has been carried out to assess the effect of wood fired oven and electric oven on food contaminants’ formation. To date, no data concerning PAHs content of Neapolitan pizza are available in literature to the best of the author’s knowledge, and therefore, it was investigated and prioritized in this contribute (data concerning acrylamide will be discussed during the oral presentation).

Flour “0”, “00” types, whole wheat flour type and the derived doughs were analyzed to test for the background PAHs levels of raw materials used for pizza production. Results revealed that all kind of flours and doughs analyzed were free from heavy PAHs. The most representative PAHs detected were light PAHs such as Ac, F and Pa, representing the 89, 85 and 92% of total PAHs in “00”, “0” and whole wheat flour types, and 80, 78 and 78% of total PAHs in doughs obtained from these flours, respectively. The $∑$ 14 PAHs was 3.4, 5 and 8.4 µg/kg d.w. in “00”, “0”, and whole wheat flour, respectively, and reached values of 9.7, 8 and 22,7 µg/kg d.w. in the doughs. These values were in agreement with the study of Ciecierska (2013) carried out on bread and different raw materials used for its baking. Figure 3 reported data concerning light PAHs content of pizza baked in wood fired oven and in electric oven. Considering a slice of pizza (as pizza it is usually consumed), the samples obtained with the whole wheat flour showed a significantly higher content of light PAHs (p<0,05), in agreement with other studies which also revealed the higher PAHs content of bran compared with other products of grain grinding (Ciecierska et al., 2013). It’s interesting to note that, for the most of light PAHs analyzed, samples with topping of tomato sauce showed higher values of PAHs compared with samples without topping. The results of pizza baked by electric oven showed no significant differences in PAHs levels in comparison with pizza baked in wood fired ovens. Also in this case, heavy PAHs were under LOD for most of the analyzed samples.

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**Figure 3.** *Content of light PAHs (µg/kg d.w.)* *of pizza baked in electric and wood-fired oven (Ac=acenaphthene; F= fluorene; Pa= phenanthrene; A=anthracene; Fl=fluoranthene; P=pyrene; F00=type “00” flour; F0= type “0” flour; FI=whole bread flour; SP= slice of pizza; R=pizza with topping of tomato sauce; B=pizza without topping; L=pizza baked in wood fired oven; E= pizza baked in electric oven).*

**5. Conclusion and future perspectives**

The high temperature reached during baking in oven can result in the formation of food processing contaminants in pizza. The obtained results showed that it is possible to contain acrylamide formation during baking by acting on the raw materials used for pizza production. The thermal treatment and raw materials, primarily flour, are responsible for the formation of light PAHs in pizza. The Commission Regulation (UE) No. 835/201 prescribed the limit of 1 µg/kg for 4 heavy PAHs (BaP, BaA, BbF, Ch) in processed cereal-based foods, not found in the investigated product.

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