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INFLUENCE OF DIFFERENT SMOKING TECHNIQUES ON CONTAMINATION BY POLYCYCLIC AROMATIC HYDROCARBONS IN TRADITIONAL SMOKED BUFFALO MOZZARELLA

Tutor

Chiar.ma Prof.ssa Teresa Cirillo

PhD Student

Valeria Urbani

Co-Tutor

Dott. Mauro Esposito

Course Coordinator. Ch.mo Prof Giancarlo Barbieri

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ABSTRACT

Technological food processing, such as grilling and smoking, can produce high levels of PAH in treated products, and risk for human health is related to the type of ingested food and the frequency of consumption. However, the recent legislation (Regulation EC 835/2011) on maximum levels of PAHs in foods did not set limits for milk and dairy products, including smoked cheeses, even if some smoking techniques can cause formation and accumulation of PAHs on food. Among smoked cheese, smoked buffalo mozzarella or "provola" is a typical product with DOP mark, it may be PAH contaminated. During traditional smoking process Mozzarella was exposed to smoke obtained from the incomplete straw or wood shavings combustion in smoking room. Alternatively, the smoked product is obtained by immersing the cheese in the so-called "liquid smoke", a solution obtained through condensation and filtration of smoke. Sometimes, illegal procedures, as corrugated paper or varnished wood combustion, are employed for smoking food production. The authorized materials are wheat straw, wood shavings of fir, beech or alder bark, which are placed in special containers.

This thesis project aimed to assess levels and distribution of B(a)P and other three marker PAHs (PAH4), as suggested by EFSA in the smoked buffalo mozzarella cheese obtained by different smoking techniques usually employed in dairies of Campania. Besides the influence of different techniques, permitted or non by law, on the content of PAHs in mozzarella was evaluated. This research, aimed to study the migration of PAHs from the external surface (rind) of the mozzarella, more exposed to the smoke, into core and so consumer exposure to these contaminants through ingestion of whole mozzarella. For this purpose were analyzed separately out of mozzarella (rind), core and whole product.

50 batches of samples of buffalo mozzarella, each of 16 samples (250 gr), produced according to the specifications established by the Decree (D.Mi.PAF. 2003), in a dairy farm located in the Salerno province (Campania) and with CE mark. Once produced, mozzarella samples were transported to another dairy, where, a mozzarella was not smoked and conceded as blank matrix while groups of three mozzarella were smoked with different techniques such as combustion of cardboard, bark of alder, chips of fir or beech, wheat straw and liquid smoke.

The obtained results showed a not PAH contamination in non-smoked mozzarella cheese samples and in the core of smoked ones.

About the rind, highest levels were found in sample obtained from cardboard combustion and in particular BaP levels ranged from 110 to 417.8 μg/kg.

However, the levels found in traditional techniques were highest in samples obtained by bark of alder combustion (5.1 μg/kg – 54.0 μg/kg), followed by chips of fir or beech combustion (1.5 μg/kg – 44.3 μg/kg) and straw (0.2 μg/kg – 28.2 μg/kg).

Considering the whole products, similar trend was observed. In particular, the use of illegal techniques such as cardboard combustion showed highest BaP (median 8.3 μg/kg), while in traditional techniques bark of alder showed a median of 2.8 μg/kg, followed by chips of fir or beech with a median 1.2 μg/kg and finally, by straw with a median of 0.4 μg/kg.

All sample obtained by immersion in liquid smoke showed not detectable PAH levels both in rind and in whole product.

A statistical analysis was carried out in order to evaluate if the time can effect on the PAH formation, and any statistical differences were found. However the use of different materials and techniques can produce a different amount of PAH in smoked food.

A statistical analysis showed that the rind can represent a barrier to migration of generated PAH into the core of the products and it so can support the accumulation of these compounds. For this reason it is recommended to remove the rind until the ingestion of this smoked cheese, considering also that our data are always higher than daily intake and intake for person evaluated by EFSA, except for liquid smoke. This suggests that mozzarella subjected to treatment with liquid smoke is preferable to the those smoked with traditional procedures.

RIASSUNTO

Processi tecnologici come la cottura alla griglia e l'affumicatura, possono determinare elevati livelli di IPA negli alimenti, ed il rischio per la salute umana dipende dal tipo di cibo assunto e dalla frequenza del consumo. Attualmente non esiste una legge che normi i livelli di IPA in latte e suoi derivati, infatti il regolamento CE 835/2011 fissa i limiti solo per alcuni alimenti. Tra i formaggi affumicati, la mozzarella di Bufala o "provola", è un prodotto tipico con marchio DOP, potrebbe essere a rischio di contaminazione da IPA. La tecnica tradizionale di affumicatura, prevede la combustione incompleta di paglia o trucioli di legno (materiali autorizzati) in appositi contenitori. In alternativa, il prodotto affumicato è ottenuto immergendo il formaggio nel cosiddetto "fumo liquido". A volte, tuttavia, sono utilizzate procedure illegali, attraverso la combustione di cartone ondulato o legno verniciato.
Il progetto di tesi si proponeva di valutare la presenza di BaP e degli altri tre IPA indicatori (PAH4) nella "mozzarella di bufala affumicata" secondo diverse tecniche di affumicatura, tra quelle maggiormente utilizzate nei caseifici campani, studiando l'influenza delle diverse tecniche, sia quelle consentite per legge, che quelle non autorizzate, sul contenuto di IPA nelle mozzarelle. Il progetto, si proponeva, inoltre, di studiare la capacità di migrazione degli IPA dalla superficie esterna delle mozzarelle, che è quella maggiormente esposta all'azione dei fumi, verso l'interno e l'esposizione dei consumatori a tali contaminanti mediante l'ingestione dell'intera mozzarella. A tale scopo sono stati analizzati separatamente l'esterno della mozzarella (pelle), il centro ed anche l'intero prodotto.
Lo studio è stato effettuato su 50 lotti di mozzarella di bufala costituiti ciascuno da 16 campioni di mozzarella di circa 250 gr, prodotte secondo le specifiche previste dal Decreto (D.Mi.PAF, 2003), in un caseificio situato nella provincia di Salerno (Campania) e a marchio CE. Una volta prodotte, le mozzarelle sono state trasportate ad un altro caseificio, dove, una mozzarella è rimasta bianca (tal quale) mentre gruppi di tre mozzarelle ciascuno sono state sottoposte a cinque differenti tecniche affumicatura quali esposizione a fumi ottenuti dalla combustione di cartone, di legno di ontano, abete e faggio, di paglia e immersione in fumo liquido.
I campioni di mozzarella non affumicata (bianco campione) non hanno mai mostrato livelli dosabili di IPA, stessi risultati si sono ottenuti dall'analisi della parte interna delle mozzarelle affumicate.
Per quanto concerne l'analisi della sola pelle, tra i campioni affumicati i più alti livelli sono sati ritrovati nelle provole ottenute per esposizione al fumo ottenuto dal cartone. In particolare i livelli di BaP nella pelle delle provole variavano da 110 a 417,8 μg/kg.
Relativamente alle tecniche di affumicatura tradizionale i più alti livelli di BaP sono stati ritrovati nella pelle delle mozzarelle affumicate per esposizione al fumo di trucioli di ontano (5,1 μg/kg – 54,0 μg/kg), seguito dai valori dei campioni ottenuti dalla combustione di legno di abete e di faggio (1,5 μg/kg – 44,3 μg/kg) e di paglia (0,2 μg/kg – 28,2 μg/kg).
Dall'analisi dei prodotti interi, comunemente consumati, è emerso lo stesso trend esposto per la pelle. In particolare, l'utilizzo di tecniche illegali quali la combustione di cartone ha permesso di rilevare i più alti livelli di BaP (mediana 8,3 μg/kg). Per quanto riguarda le tecniche tradizionali i più alti livelli di BaP sono stati ritrovati nei campioni ottenuti per combustione di legno di ontano (mediana 2,8 μg/kg) e di abete e faggio (mediana 1,2 μg/kg), mentre i campioni ottenuti dalla combustione della paglia hanno mostrato una mediana di 0,4 μg/kg.
In nessun campione di mozzarella affumicata per immersione in" fumo liquido", sono stati riscontrati apprezzabili livelli di IPA, né sul totale né sulla pelle.
Dall'analisi statistica condotta è emerso che il tempo non influisce sulla formazione di tali composti. Sicuramente l'utilizzo di materiali diversi può però essere considerato determinante circa la produzione di IPA.
Inoltre confrontando i livelli di tali contaminanti ottenuti dall'analisi della sola pelle e della parte esterna e valutando i risultati ottenuti dall'analisi statistica condotta circa tali risultati, è emerso che la pelle esposta ai fumi di combustione sembrerebbe funzionare da barriera contro la migrazione degli IPA dall'esterno verso l'interno del prodotto, favorendo l'accumulo di tali composti. Per tal motivo sarebbe auspicabile

rimuovere la pelle prima del consumo di tale alimento, anche perché i nostri risultati circa ingestione quotidiana e per persona risultano sempre superiori a quelli ritrovati dall'EFSA, eccetto per il fumo liquido.
Questo suggerisce inoltre che l'affumicatura per immersione in "fumo liquido" è preferibile rispetto alle altre tecniche.

0. Preface

As suggested by Cirillo et al., 2012, the contamination of food by chemical hazards is of worldwide public health concern and is a leading cause of trade problems internationally.

Contamination may occur through environmental pollution of the air, water and soil or through the intentional use of various chemicals or from food treatments, processing, storing and cooking methods. Among organic contaminants, polycyclic aromatic hydrocarbons (PAHs) represent an important class of food contaminants and the diet represents the most important way of exposure for non-occupationally exposed populations (European Food Safety Authority (EFSA) 2005). The occurrence of PAH in foods is from environmental pollution and processing procedures. While the transportation of PAHs in the atmosphere is influenced by their volatility, in the terrestrial environment the distribution of PAHs is influenced by their lipophility and solubility in water which determine their capacity for transport and distribution between the different compartments, their uptake and accumulation by living organisms, and their bioaccumulation in the food chain, in particular in lipid tissue of plants and animals. Low molecular mass PAHs can be mainly concentrated by adsorption through the waxy surface of vegetables and fruits. PAH concentrations are generally greater on a plant's surface (peel, outer leaves) than in the internal tissues. Careful washing may remove up to 50% of the total surface adsorbed PAHs (Edwards 1983; Nielsen et al. 1996). In the Cirillo study (2012) it was explained the average background values are usually in the range 0.01–1.0 mg kg^{1} in uncooked foods (Guillen et al. 1997; Phillips 1999). Processing procedures, such as smoking and drying, and cooking of food are commonly thought to be the major sources of contamination by PAHs (Chen and Lin 1997). Levels as high as 200 $mgkg^{1}$ have been found for individual PAHs in smoked fish and meat (European Commission 2002). Jira (2004) found benzo[a]pyrene concentrations ranging from 0.05 to 0.35 $mgkg^{-1}$(mean¼0.12 $mgkg^{-1}$) in smoked ham and sausage.

Duedahl-Olesen et al. (2006) reported benzo[a]pyrene levels for Danish smoked products such as bacon, small sausages and salami below 0.6 $mgkg^{-1}$. Cooking practices, such as grilling, frying, and roasting, can influence the production of PAH in the food, the type, number and amount of which depend on various parameters as temperature, time, kind of fuel used, distance from the heat source, drainage of fat, etc. (Saint-Aubert et al. 1992; Mottier et al. 2000). Lodovici et al. (1995) found in foods typical of the Italian diet values for PAHs (nine compounds) ranging from 0.52 to 42.00 mgkg–1. In barbecued meat, total PAHs were found to be present at levels up to 164 $mgkg^{-1}$, with benzo[a]pyrene present at levels as high as 30 $mgkg^{-1}$ (Phillips 1999). In Europe, an average benzo[a]pyrene
concentration of 0.2 $mgkg^{-1}$ was reported for fresh fish and ranging from 1.4 to 5.3 $mgkg^{-1}$ for smoked fish obtained, respectively, by unknown or traditional smoking methods (European Commission 2004).
Contamination of vegetable oils (including olive residue oils) with PAHs usually occurs during technological processes such as direct fire drying, where combustion products may come into contact with the oil seeds or oil (Larsson et al. 1987; Speer et al. 1990; European Standing Committee on Foodstuffs 2001)

0.1 References

T. Cirillo, P. Montuori, P. Mainardi, I. Russo, E. Fasano, M. Triassi and R. Amodio-Cocchieri. Assessment of the dietary habits and polycyclic aromatic hydrocarbon exposure in primary school children. Part A, 27:7, 1025-1039 http://dx.doi.org/10.1080/19440041003671262

Chen BH, Lin YS. 1997. Formation of polycyclic aromatic hydrocarbons during processing of duck meat. J Agric Food Chem. 45:1394–1403.

Duedahl-Olesen L, White S, Binderup ML. 2006. Polycyclic aromatic hydrocarbons (PAH) in Danish smoked fish and meat products. Polyc Arom Comp. 26:163–184.

Edwards NT. 1983. Polycyclic hydrocarbons (PAHs) in the terrestrial environment. A review. J Environ Qual.12:427–441.

European Standing Committee on Foodstuffs. 2001. Outcome of the expert group meeting on 3 October on ways to prevent contamination of olive residue oil and other oils with polycyclic aromatic hydrocarbons (PAH).
Summary record of the 85th meeting of the Standing Committee on Foodstuffs, 25 October 2001, agenda item9. Available from: http://ec.europa.eu/food/fs/rc/scfs/rap09_en.pdf/

European Commission. 2002. Opinion of the Scientific Committee on Food on the risks to human health of polycyclic aromatic hydrocarbons in food expressed on 4 December 2002. Brussels (Belgium): European Commission. Available from http://europa.eu.int/comm/food/fs/fc/scf/index_en.htlm/

European Commission. 2004. Report on Tasks for Scientific Cooperation (SCOOP). Collection of occurrence data on polycyclic aromatic hydrocarbons in food. Report of experts participating in task 3.2.12. Brussels (Belgium):European Commission.: http://europa.eu.int/comm/food/food/chemicalsafety/contaminants/out153_en.pdf/

European Food Safety Authority (EFSA). 2005. Opinion of the Scientific Committee on a request from EFSA related to a harmonized approach for risk assessment of substances which are both genotoxic and carcinogenic.
EFSA J. 282:1–31.
http://www.efsa.europa.eu/EFSA/Scientific_Opinion/sc_op_ej282_gentox_en3,0.pd

Jira W. 2004. A GC/MS method for the determination of carcinogenic polycyclic aromatic hydrocarbons (PAH) in smoked meat products and liquid smokes. Eur Food Res Technol. 218:208–212.

Lodovici M, Dolara P, Casalini C, Ciappellano S, Testolin G. 1995. Polycyclic aromatic hydrocarbon contamination in the Italian diet. Food Addit Contam.12:703–713.

Mottier P, Parisod V, Turesky RJ. 2000. Quantitative determination of polycyclic aromatic hydrocarbons in barbecued meat sausages by gas chromatography coupled to mass spectrometry. J Agric Food Chem. 48:1160–1166.

Phillips DH. 1999. Polycyclic aromatic hydrocarbons in the diet. Mutat Res. 443:139–147.

Saint-Aubert B, Cooper JF, Astre C, Spiliotis J, Joyeux H. 1992. Evaluation of the induction of polycyclic aromatic hydrocarbons (PAH) by cooking on two

geometrically different types of barbecue. J Food Compos Anal.5:257–263.
Speer K, Steeg E, Horstmann P, Ku¨ hn Th, Montag A. 1990. Determination and distribution of polycyclic aromatic hydrocarbons in native vegetable oils, smoked fish products, mussels and oysters, and bream from the river
Elbe. J High Resol Chromatogr. 13:104–111.

1. State of the art

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds characterized by two or more condenses aromatic rings and formed mainly by incomplete combustion or decomposition of organic matter induced by heat. In particular, the name PAHs refers to compounds containing only carbon atoms and hydrogen (ie, PAHs and alkyl-PAHs), while the more general name "polycyclic aromatic compounds" also includes the functional derivatives (eg. nitro-PAHs) and heterocyclic analogues (eg. aza-PAHs).PAHs are commonly found in air, water, soil and food. They are produced in natural events or human activities besides the human activities regard [IPCS, 1998; Organization, 2008]:

- Industrial actions (eg aluminium production, incinerators): processing of coal and oil

- motor vehicles emissions

- Combustion in agriculture

- Tobacco smoke

- Domestic heating of wood and coal

- Cooking food on flame

The natural sources are mainly represented by plants and bacteria biosynthesis and natural events such as volcanic action. They are ubiquitous contaminants, and the main source of them for human is represented by food. Food contamination can regard non-processed or processed food. Presence of PAHs in non-processed food is essentially caused by exposure to contaminated atmospheric (eg. on wheat, fruits and leafy vegetables), and by the contaminated soil (eg. Potatoes), absorption by water of river and sea (eg. mussels, fish, shellfish).

Therefore the presence of PAHs in food may be due to environmental contamination and contamination through the food chain. In addition, food can accumulate PAHs during cooking food at high temperatures (grilled foods, smoked, fried) or during technological processes such as heating, drying and smoking, which if not done correctly determine the pyrolysis of proteins, lipids and carbohydrates, and therefore the products of combustion come into direct contact with the food. These processes are intended to increase the shelf life of the food or to give it colour, flavour and aroma particular. [Garcia,1999; Falcó G.,2003; Garcia-Falcon M.S.,2005; Duedahl-Olesen L.,2010]

PAHs can effect human health and for their proven carcinogenicity, and for their mutagenic effects. Benzo[a]Pyrene (Figure 1) one of the more investigated for its toxicological profile and it is usually evaluated in environmental and food.

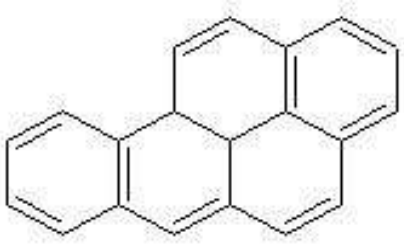


Figure 1 - Chemical structure of Benzo[a]Pyrene

It is frequently used as an indicator of the class of IPA, as regards both the levels of

contamination that the carcinogenic risk.

1.2 Chemistry of PAHs

PAHs are hydrocarbons consisting of two or more condensed benzene rings, in a planar structure. These rings may be alkyl, cycloalkyl or heterocyclic-substituted. It is possible to obtain 22 different hydrocarbons from condensation of five benzene rings; possible combinations became 88 considering 6 rings and 333 when having 7 ones. Obviously not all of these compounds are present in the environment in so much quantity as to be considered pollutants. More attention are: Benzo[a]anthracene, Benzo[b] fluoranthene, Benzo[j]fluoranthene, Benzo[k]fluoranthene, Benzo[g,h,i]perylene, Chrysene, Cyclopenta[c,d]pyrene, Dibenzo[a,h]anthracene, Dibenzo[a,e]pyrene, Dibenzo [a,h]pyrene, Dibenzo[a,i]pyrene, Dibenzo[a,l]pyrene, Indeno[1,2,3-c,d]pyrene and 5-methylchrysene. The simplest-structured PAH is Naphthalene (Figure 2), characterized by benzene rings and it is in gaseous state. Naphthalene has a planar cyclic structure constituted by a sequence of sp2 hybridized carbon atoms.

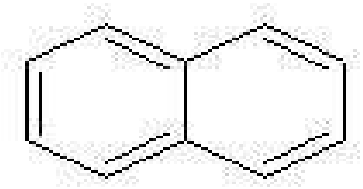


Figure 2 - Molecular Structure of Naphthalene

Considering PAHs are classified as:

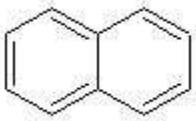
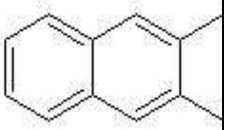
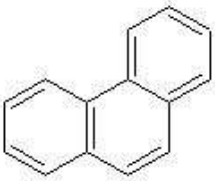
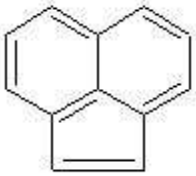
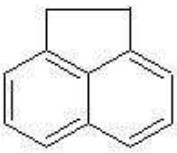
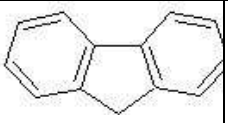
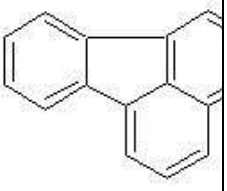
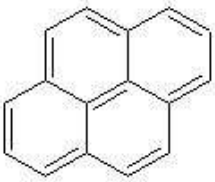
• Light PAHs: constituted by two or three rings;

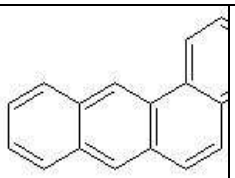
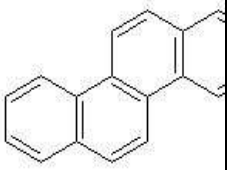
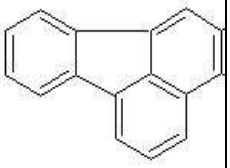
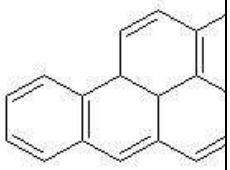
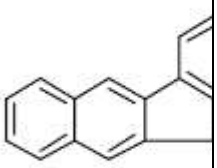
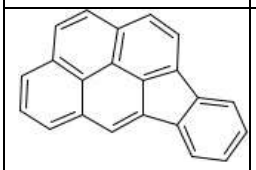
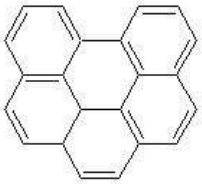
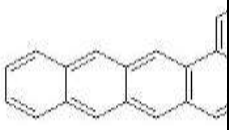
• Heavy PAH: constituted by four or more rings.

Compounds containing less than four rings generally remain in gaseous state when they are released into the atmosphere, and about after twenty-four hours are usually degraded. PAHs with more than four rings are generally bound to particulate and their stability is determined by molecular structure; generally linear structure is less stable.

PAHs are poorly soluble or completely insoluble in water; the solubility decreases with molecular weight increasing. They are solid at room temperature and have both high melting and boiling points. In the purest form PAHs are solid and transparent, white-coloured or ranging from pale yellow to pale green.

Table 1 - Chemical characterization of the 16 PAHs included in the list of EPA

Structure	Composed weight	Molecular Point	Merger (°C) Point	Boiling Point (°C)
	Naphthalene $C_{10}H_8$	128,16	79	218
	Anthracene $C_{14}H_{10}$	178,24	216	340
	Phenanthrene $C_{14}H_{10}$	178,24	101	338
	Acenaphthylene $C_{12}H_8$	152,20	93	280
	Acenaphthene $C_{12}H_{10}$	154,21	95	279
	Fluorene $C_{13}H_{10}$	166,22	116	295
	Fluoranthene $C_{16}H_{10}$	202,26	111	383
	Pyrene $C_{16}H_{10}$	202,26	156	393

	Benzo[a]Anthracene $C_{18}H_{12}$	228,30	162	435
	Chrysene $C_{18}H_{12}$	228,30	256	441
	Benzo[b]Fluoranthene $C_{20}H_{12}$	252,32	168	481
	Benzo[a]Pyrene $C_{20}H_{12}$	252,32	177	496
	Benzo[k]Fluoranthene $C_{20}H_{12}$	252,32	217	480
	Indeno[1,2,3-cd]Pyrene $C_{22}H_{12}$	276,34	163,6	536
	Benzo[g,h,i]Perylene $C_{22}H_{12}$	276,34	278	550
	diBenzo[a,h]Anthracene $C_{22}H_{14}$	278,36	270	524

Aromatic rings in the structures give them low reactivity and lipophilic character. A measure of lipophilicity is given by the water / n-octanol partition coefficient (K_{ow}), which expresses the accumulation capacity of the compounds in apolar phases such as the lipid tissues of organisms. The K_{ow} is determined by the following formula:

K_{ow} = [X] octanol / [X] water

where [X] corresponds to the concentration in moles / L of substance X. Logarithmic function of K_{ow} is often more used than simple K_{ow}; in fact, the EPA (Environmental Protection Agency) states that the compounds that have a $\log K_{ow}$ value of above 3.5 should be considered potentially dangerous to the environment. Benzo [a] Pyrene, for example, has a $\log K_{ow}$ value of 6.3.

1.3 Mechanism of formation

PAHs are produced during combustion process. The mechanism of formation is mainly due to the repolymerization of hydrocarbon fragments formed during the "cracking", ie the production of many fragments from fuel molecules in contact with the fire. The reaction of repolymerization occurs especially in conditions of oxygen deficiency; in general the lower oxygen ratio induces higher rate of PAHs formation: during the different step of the reaction, the fragments often lose some hydrogen atoms generating water by combination with oxygen. The originated radicalic molecules which necessarily recombine to give larger and more stable compounds such as PAHs; this step is called "pyro synthesis".

After cracking process and partial combustion there is a prevalence of radical fragments containing two carbon atoms which can react with a molecule of acetylene to give a radicalic molecule with 4 carbon atoms. The resulting radical can subsequently react with another molecule of acetylene and cyclize in a stable six atoms ring. The radicalic cycle can bind further acetylene molecules originating side chains that finally give condensed benzene rings (Figure 3).

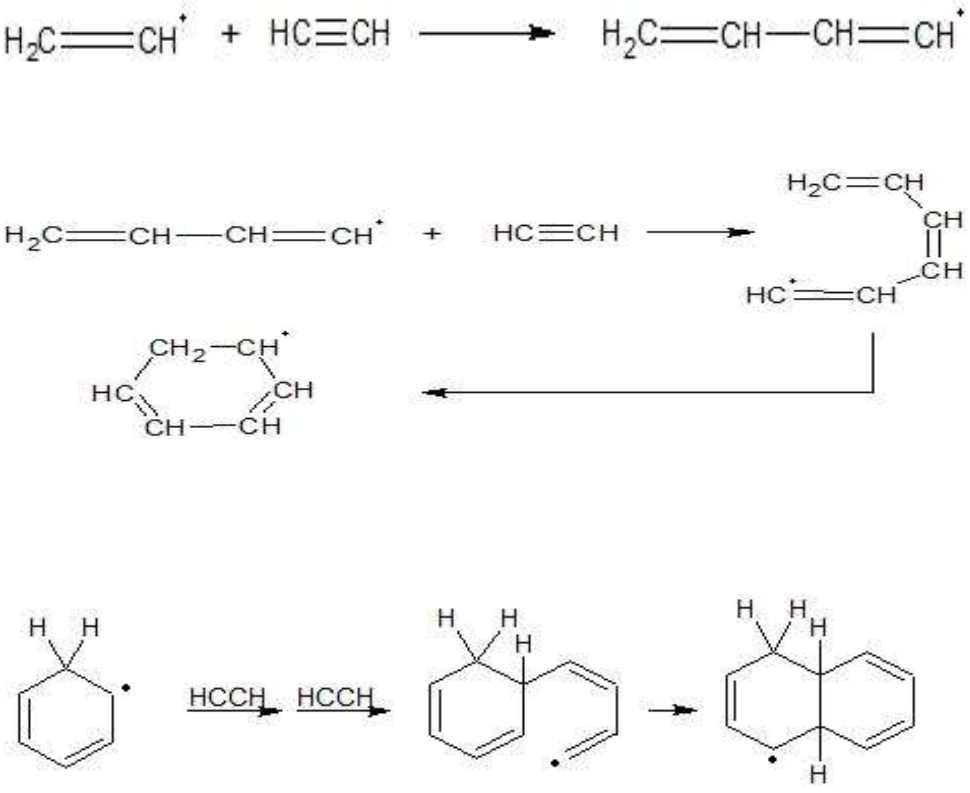


Figure 3 - Mechanism of formation of PAHs

High temperatures (650-900 ° C) and low oxygen concentration promote the formation of PAHs; or they are produced during combustion of organic material at low temperatures (100-300 ° C) but for long time. During pyro synthesis, lighter PAHs quickly react together so molecular weight increases, as well as volatility and water solubility decrease, while boiling and melting points increase. These are the reasons for which PAHs with a greater number of aromatic rings are more thermodynamically stable and therefore more persistent. Contrary benzene, in which all six carbon atoms have identical reactivity, in the polycyclic aromatic hydrocarbons atoms different C atom positions in rings produce a instability, both during oxidation and aromatic electrophilic substitution reactions.

1.4 Toxicity of PAHs

The PAHs toxicity is showed in the human body when during their metabolites [Baird WM1., 2005], they are converted in metabolites soluble in water with carcinogens effects. On the working group of the International Agency for Research on Cancer [IARC International Agency for Research on Cancer, (2010)] evaluating a lot of studies on human exposure to PAHs through diet, suggested association between consumption of PAH by foods and increased risk of colon and rectal and pancreas adenomas.

In particular IARC established carcinogenetic class for some PAHs as showed a table 1.

The IARC classification is reported in Table 2.

Table 2 - Classification of carcinogenicity of some IPA [IARC]

Substance	Class
Benzo[a]pyrene	2A
DiBenzo[a,h]anthracene	2A
Benzo[a]anthracene	2A
Benzo[b]fluoranthene	2B
Benzo[K]fluoranthene	2B
Chrysene	2B
Indeno[1,2,3,c,d]pyrene	2B
Benzo[g,h,i]perylene	3
Anthracene	3
Fluoranthene	3
Fluorene	3
Fenanthrene	3
Pyrene	3

Legend: = 2A Probable carcinogenic effect in humans; 2B = Possible carcinogenic effect in humans; 3 = Not carcinogenic effect in humans

Among these PAHs, BaP is frequently used as a marker of the presence of PAHs considering that there is a substantial similarity, (at least in terms of order of magnitude), between the "profiles" of PAHs and BaP (ie the relationship between the PAHs concentrations, especially carcinogenic ones, and BaP concentration), also observed in samples of different source; however the carcinogenic potency of BaP is significantly higher than other PAHs[IPCS, 1998]. The metabolic pathway of BaP is summarized in Figure 4.

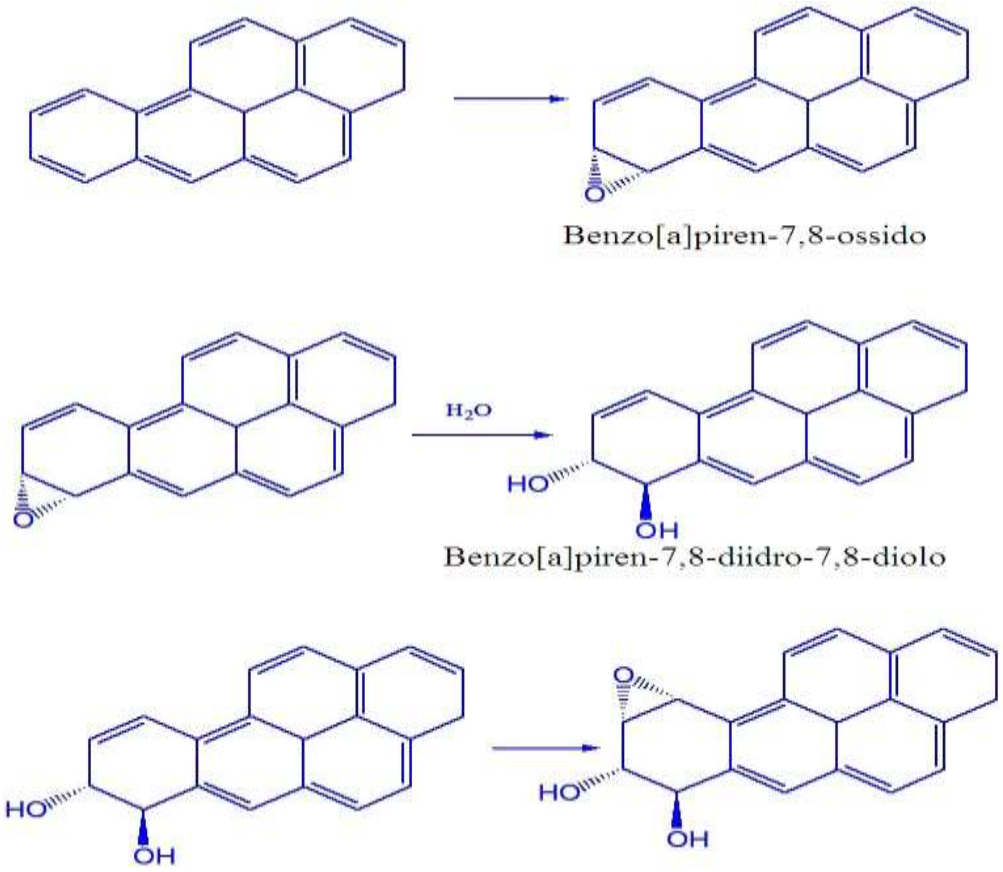


Figure 4 - BaP metabolic pathway

BaP undergoes epoxidation catalyzed by cytochrome P450, in locations 7 and 8, that are the most reactive and represent the so-called region K. Subsequently the epoxide undergoes a nucleophilic addition by water, giving thus the formation of a diol, more water-soluble and therefore more easily eliminated. This metabolite can be further metabolized by cytochrome P450 to form a diol epoxide. A diffused idea is that the diol epoxide is the species responsible of the DNA-binding through nucleophilic addition, for example, of adenine (Figure 5). The covalent attachment of the large hydrocarbon residue is an obvious DNA damage that causes mutations and a higher probability of carcinogenicity [R.E. Lehr, D.M. Jerina, 1977].

The actual carcinogen is one of the four theoretically possible diastereomers of benzo[a] pyrene-7,8-diol-9-10-epoxide, i.e. that with 7R, 8R, 9R, 10R configuration.

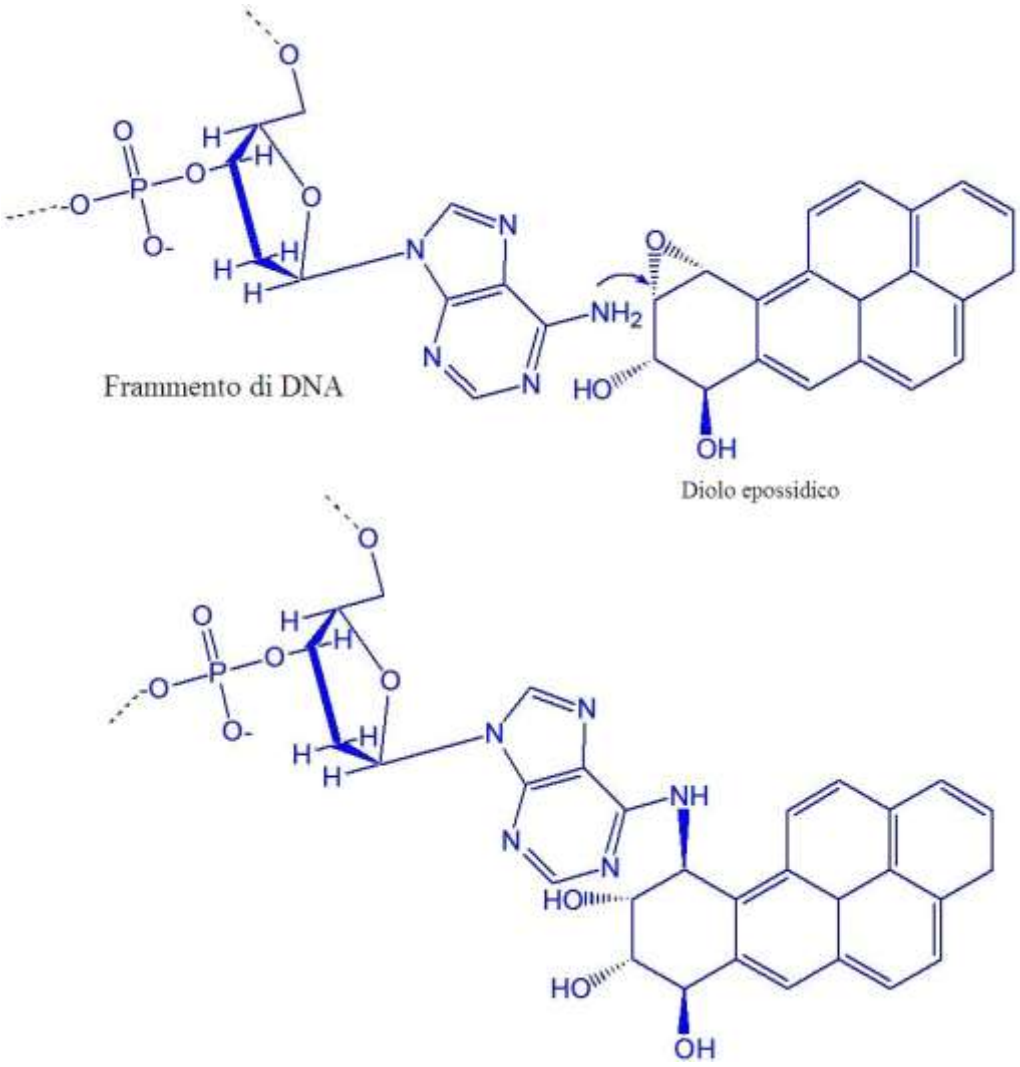


Figure 5 - Reaction of B (a) P-7,8-diol-9,10-epoxide with DNA

Another way of detoxification of these compounds is represented by conjugation with glutathione.This reaction is catalyzed by glutathione S-transferase in liver, lung and kidney. In this case, metabolism is quite fast and PAHs are eliminated within a few days through hepatobiliary excretion, urine and feces.

Usually, human exposure involves complex mixtures of PAHs in which are present other carcinogens. So the carcinogenicity profile overlaps with genotoxicity profile both for single PAH and for complex mixtures. This highlights the functional association between damage caused by DNA adducts, induction of mutations and long-term carcinogenic effects of PAHs [Nesnow S., 1995]. Always on the basis of experimental toxicology, BaP is defined as "promoter", i.e. the substance that reacts with DNA, causing irreversible effects conveyed to progeny through cellular division.

BaP is therefore also genotoxic and mutagenic according to the processes shown in figure 6.

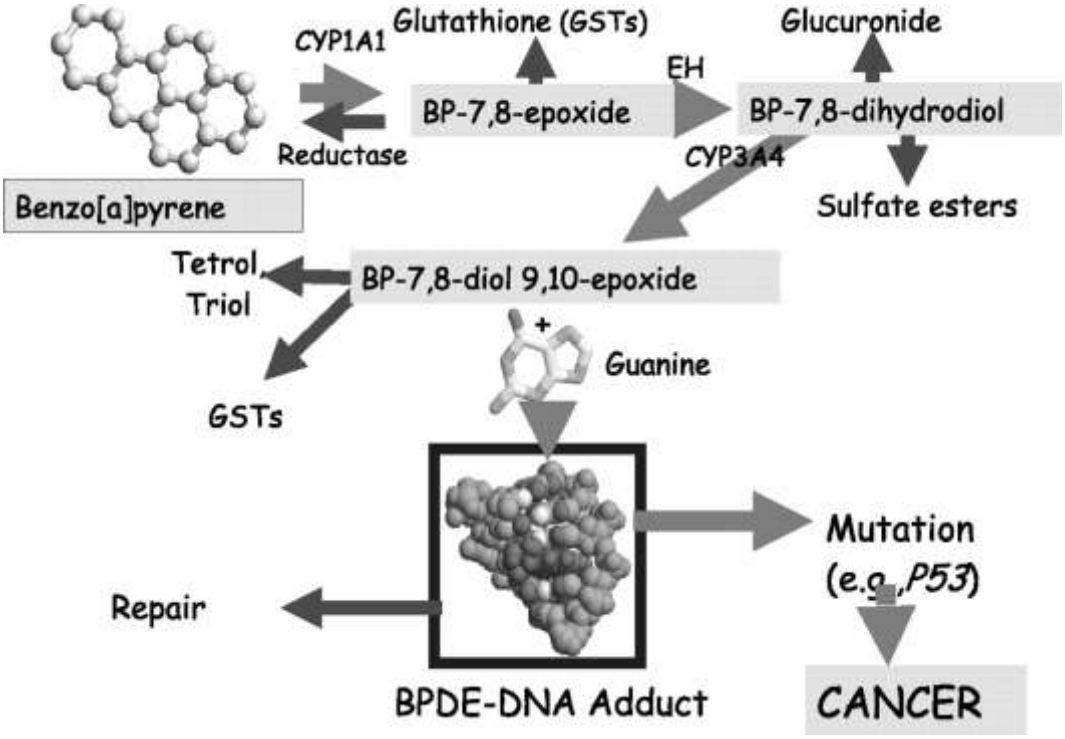


Figure 6 - Diagram of the genotoxicity and carcinogenicity of BaP

The carcinogenicity of PAHs is notoriously associated with the presence of K-regions or bay-regions. In animal experimentals it has been noted that the relative position of the condensed rings play an important role in determining the level of potential carcinogenicity. The PAHs that represent the most potent carcinogens possess a cavity region (bay-region) constituted by a branching in the sequence of atoms of the benzene rings (Figure 7). The high reactivity of these compounds regards cytochrome P-450 is related to the olefinic nature of the unsaturation on peripheral benzilic ring.

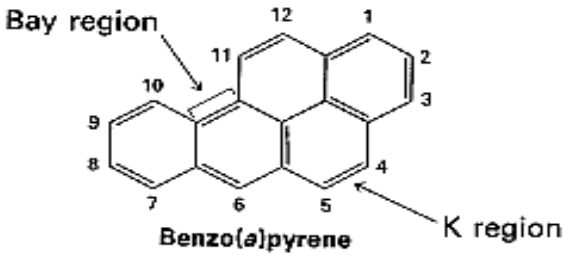


Figure 7 – Bay region and k-region of BaP

PAHs manifest their toxicity on multiple organs and tissues; thanks to their high solubility, they can cross easily the membrane, with active transport. PAHs exposures related to cancers of the lung, bladder, and skin, but also of kidney, larynx, breast; therefore, a lot of other kind of tumours are suspected to be promoted

by PAHs [ATSDR (1995)].

PAHs may also interfere with the functionality of the cellular membrane by reversibly binding to lipophilic sites. They cause immunosuppression and abnormal alteration in blood compartment, damages to the pulmonary system and the reproductive one.

Once ingested or inhaled, PAHs are rapidly absorbed through the gastrointestinal tract or the lung epithelium respectively and distributed in various tissues (especially in more lipidic ones), including fetal. The preferred accumulation tissues are breast, pancreas, and, in a particular fatty tissue; the intestinal tract contains high concentrations of PAHs even when are absorbed through other way, because of biliary excretion and entrapment in respiratory tract mucus (which is then swallowed). The organs rich in fatty tissue may act as reserves from which PAHs are gradually released.

In the past decade, the effects deriving from PAH ingestion have been evaluated by the International Programme on Chemical Safety (IPCS) (World Health Organization (WHO)/IPCS 1998), the Scientific Committee on Food (SCF) (European Commission 2002), and by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO 2005). The SCF concluded that for 15 PAHs, (benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]-fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, cyclopenta[c,d]pyrene, dibenz[a,h]anthracene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]-pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-cd]pyrene and 5-methylchrysene clear evidence exists of mutagenicity/ genotoxicity in somatic cells in experimental animals in vivo and of carcinogenic effects in various types of bioassays in experimental animals. These compounds may be consequently regarded as potentially genotoxic and carcinogenic to humans and considered a priority group in the assessment of the risk of long-term adverse health effects following dietary intake of PAHs. Benzo[a]pyrene was suggested as a marker of occurrence and effect of the carcinogenic PAHs in food, based on an examination of the PAH profiles in food. Furthermore, studies on experimental animals showed various toxic effects, such as haematological effects, liver, reproductive and developmental toxicity, and immunotoxicity for other PAHs (acenaphthene, anthracene, fluoranthene, fluorene, naphthalene, pyrene) with no observed adverse effect levels (NOAELs) ranging from 70 to 1000 mgkg_1 bw day_1 in subchronic studies (European Commission 2002). In 2007, the EFSA Panel on Contaminants in the Food Chain reviewed the available data on the occurrence and toxicity of PAHs, highlighting that about 30% of all the foods analysed were negative for benzo[a]pyrene but positive for other carcinogenic and genotoxic PAHs. Eight PAHs, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, benzo[a]pyrene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene, individually or in combination, were considered to be currently the only possible indicators of the carcinogenic potency of PAHs in food. As an alternative to the NOAEL and lowest observable adverse effect levels (LOAEL) approach, a new limit dose that provides a more quantitative alternative to the first step in the dose–response assessment, the bench mark dose (BMD), was proposed, according to what suggested by Crump (1984). The BMD lower limit (BMDL) is the BMD lower confidence limit for a 10% increase in the number of tumour-bearing animals compared with control animals (BMDL10). According to EFSA, the CONTAM Panel calculated the BMDL10 values for benzo[a]pyrene; the sum of benzo[a]pyrene and chrysene (PAH2); the sum of PAH2, benz[a]anthracene and benzo[b]- fluoranthene (PAH4); the sum of PAH4, benzo[k]fluoranthene, benzo[ghi]perylene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene (PAH8) using a range of statistical models, and selected the lowest BMDL10 values from the statistical models that fit the data adequately. These values are 0.07; 0.17; 0.34 and 0.49mg kg^{-1} bw day^{-1}, respectively. The CONTAM Panel defined a Margin of Exposure (MOE) approach based on dietary exposure to benzo[a]pyrene, PAH2, PAH4 and PAH8 for average and high-level consumers and their corresponding BMDL10 values (EFSA 2008) (Cirillo et al., 2012).

1.5 European Regulations in food security area regarding PAHs

Regulation (EC) No. 1881/2006 [Regolamento della Commissione Europea 1881/2006] as amended by Regulation (EC) No. 835/2011 [Regolamento della Commissione Europea (EU) 835/2011] of the Commission of 19 December 2011, establishes maximum levels of PAH in infant and early childhood food, in oils and fats, smoked meats, fish muscle (smoked or not), crustaceans, cephalopods and bivalves, but not for dairy products. Initially, the Regulations established the use of Benzo[a]pyrene, as a marker for the occurrence and effect of carcinogenic PAH in food, setting maximum levels in mg / kg of wet weight, but at the same time suggesting the determination of other PAHs in foods through monitoring plans. Consequently, with the Regulation (EC) No. 835/2011, it has been established maximum residue levels not only for Benzo[a]pyrene, but also for the sum of four PAHs: Benzo[a]pyrene, Chrysene, Benzo[b] Fluoranthene and Benzo[a]anthracene (Table 3).

At the same time, the Regulation (EC) No. 836/2011 [Regolamento della Commissione Europea (EU) 836/2011] amending Regulation (EC) 333/2007, establishes sampling and analysis methods for official control of the level of benzo(a)pyrene in foodstuffs.

Table 3 - Maximum admitted levels of PAHs in foods of animal origin

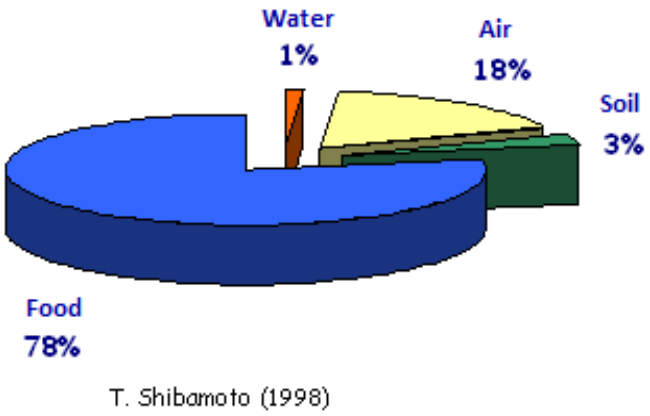
Product	Maximum content of BaP(µg/kg wet weight)	Maximum content of the sum of 4 PAHs(µg/kg wet weight)
Oils and fats for human nutrition for direct consumption or use as an ingredient in food	2.0	10.0
Smoked meats and smoked meat products	5.0 (till 31.8.2014) 2.0 (since 1.9.2014)	30.0 (since 1.9.2012 till 31.8.2014) 12.0 (since 1.9.2014)
Muscle meat of smoked fish and smoked fishery products, excluding bivalve molluscs	5.0 (till 31.8.2014) 2.0 (since 1.9.2014)	30.0 (since 1.9.2012 till 31.8.2014) 12.0 (since 1.9.2014)
Smoked sprats and canned smoked sprats (Sprattus sprattus); bivalve molluscs (fresh, chilled or frozen;	5.0	30.0

meat and meat based products from heat-treated and sold to the final consumer		
Bivalve smoked	6.0	35.0
Foods processed from cereals and infants and young children foods	1.0	1.0
Infant formulas and follow-on formulas, including infant milk and follow-on milk	1.0	1.0

1.6 Occurrence of PAHs in food

Various studies have shown that intake of PAHs occurs mainly through diet [Bocca, B.2003]. In particular, a study conducted in Italy [Lodovici, M. 1995] showed that the total PAHs assumption through food is around 3µg per day, while for carcinogenic PAH,the intake range from 1-4 g per day. These results are higher than intake due to inalation which is about 370 ng per day for total PAHs and 130 ng per day for carcinogenic PAH. The leaf vegetables such as lettuce and spinach, can represent a major source of carcinogenic PAH, because these substances transported by atmospheric particulates can drop off on leaf in some cases they can derive from contaminated soil contact. The presence of PAHs in mussels, fish and shellfish can result from absorption by contaminated water, both river and sea. Foods can accumulate PAHs during cooking process at high temperatures (eg: grilled foods, smoked, fried) or during technological processes such as heating, drying and smoking [Simko, P. 2005; Suchanovà, M. 2008]. Larsson [Larsson, B.K. 1983], was found in grilled meat products just direct contact between food and flame gives significant productions of PAHs (up to 212 ppb of Benzo[a]pyrene). Smoking is one of the oldest techniques of food preservation which also has a favorable effect on sensory properties, in particular on taste, color and texture.

Figure 8 - Percentage of exposure to carcinogenic PAHs from Water, Air, Soil and Food



1.7 PAH contamination in process: smoking technique

Among the smoked foods, cheeses are considered as more tasty by consumer and in particular for buffalo mozzarella is required smoked version.

Buffalo mozzarella cheese is a typical products obtained the DOP (Appellation of Protected Origin) mark with the CEE regulation n. 1107 of 12 June 1996 for the identification of safeguarded food. Mozzarella di bufala Campana DOP is a cheese produced with whole buffalo milk and regulated by a disciplinary emanated by Decree 28 September 1979. In order to safeguarding mozzarella all over the world it was created "Consorzio per la tutela del formaggio mozzarella di bufala Campana" (Consortium for safeguarding of Campania's buffalo mozzarella cheese), that promote, safeguard typicality and peculiar characteristics of mozzarella. In fact, buffalo mozzarella must be exclusively produced with buffalo milk, from animals bred in allowed areas of Campania and south Lazio, and must be produced in the same areas.

Frequently buffalo mozzarella is subjected to a smoking process, for increasing shelf-life and giving cheese aroma and flavour. This process, as before described, could produce toxic and carcinogenic PAHs, that can affect human health.

The traditional smoking technology consists in an incomplete combustion of straw or wood chips placed in smoking room [Guillén, M.D. 2000; Guillén, M.D. 2011], usually only an iron box, while in some cases there is an oven equipped with display in order to adjust the temperature and the exposure time. The authorized materials for smoking range from wheat straw to dry poplar bark, alder wood flakes. The smoking process may also be carried out through the immersion of the product in the so-called "liquid smoke", an aqueous solution obtained from the smoke produced by wood combustion, then filtered (EU REG. 2065/2003).

The pyrolysis of wood takes place in smoking room (300-400 ° C), and smoke may be conveyed in a condensation tower where it meets an upstreaming flow of water. The water captures molecules present in the smoke and then fall into a settling tank where oil and tar components are removed by decanting to be recycled. The aqueous flavored mixture is filtered on carbon and bubbled in cold water, purified by extraction and further filtration just to obtain a thick liquid extremely flavored but with a very low concentration of PAHs (0.1-1 µg / kg).

Smoking, or rather what it comes from its processing, is collected in vegetable oils and diluted before marketing (1-3%) thus broking down, further concentration of PAHs.

It is know that smoking, if made without following the correct techniques is contamination by polycyclic aromatic hydrocarbons [Garcia-Falcon M.S. 1999; Duedahl-Olesen L. 2010; Guillén M.D. 2000; Riha W.E 1992; Aydinol P. 2013; Ozcan T. 2011; Michalski R. 2003]. The risk of contamination increases if there are techniques and materials employed not permitted during smoking process. However, illegal materials for combustion, in particular various kinds of papers or varnished woods are widely used for this smoking technique.

1.8 Human exposure

Dietary intakes

It is possible to estimate human exposure to PAHs through ingestion of contaminated food. According to what is described by EFSA, estimation of exposure is done by multiplying the median concentration of PAHs in a food for the median of its daily consumed quantity, obtained from consumption tables of national and international companies. Furthermore, to get an accurate evaluation of consumer exposure, it is necessary to divide this result by the individual average body weight, more or less equal to 60 Kg (EFSA, 2008). EFSA suggests to make these estimates of exposure by considering markers PAHs, ie BaP, PAH2 (Benzo[a]Pyrene + Chrysene) and PAH4 (PAH2 + Benz[a]Anthracene + Benzo[b]Fluoranthene).
EFSA in a report in 2008 has collected from member states data regarding the medians of consumption of different food categories and the related median values of BaP, PAH2 and PAH4 calculated in ng/day (Table 4)

Table 4: Consumer exposure to benzo[a]pyrene (BaP), PAH2 and PAH4 for each food category for which occurrence data are available. The median value of the mean consumption reported by the Member States for consumers only was calculated.

CATEGORY	CONSUMPTION MEDIAN g/day	BaP ng/day	PAH2 ng/day	PAH4 ng/day
Cereals and cereal products	257	67	129	257
Sugar and sugar products including chocolate	43	5	13	25
Fats (vegetable and animal)	38	26	112	177
Vegetables, nuts and pulses	194	50	124	221
Fruits	153	5	40	75
Coffee, tea, cocoa (expressed as liquid)	601	21	55	106
Alcoholic beverages	413	4	12	25
Meat and meat products and substitutes	132	42	107	195
Seafood and seafood products	27	36	140	289
Fish and fishery products	41	21	84	70
Cheese	42	6	12	20

EFSA reported that the median BaP, PAH2, PAH4 dietary exposures, which referred to an adult of 60 kg bw and which were calculated for mean dietary consumers, across European countries are for BaP 3.9 ng kg $^{-1}$ bw day $^{-1}$; for PAH2 10.7 ng kg $^{-1}$ bw day $^{-1}$; for PAH4 19.5 ng kg $^{-1}$ bw day $^{-1}$. In particular considering only cheese intake the values are for BaP 0.1 ng kg $^{-1}$ bw day $^{-1}$; for PAH2 0.2 ng kg $^{-1}$ bw day $^{-1}$; for PAH4 0.3 ng kg $^{-1}$ bw day $^{-1}$.

1.9 References

Aydinol P. and Ozcan T. (2013) The effect of natural and liquid smokes on the benzo[a]pyrene content and quality parameters of Circassian cheese. International Journal of Dairy Technology 66, Issue 4, 498–504.

ATSDR (1995). Chemical and Physical Information, in: Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs), ATSDR, Atlanta, Georgia, USA, pp. 209-221.

Baird WM1, Hooven LA, Mahadevan B. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. Environ Mol Mutagen. 2005 Mar-Apr;45(2-3):106-14.

Bocca B., Crebelli R., Menichini E. (2003). Presenza degli idrocarburi policiclici aromatici negli alimenti. Istituto Superiore di Sanità. Rapporti ISTISAN 03/22.

Cirillo T., Montuori P., Mainardi P., Russo I., Fasano E., Triassi M. and Amodio-Cocchieri R. Assessment of the dietary habits and polycyclic aromatic hydrocarbon exposure in primary school children. Part A, 27:7, 1025-1039 http://dx.doi.org/10.1080/19440041003671262

Duedahl-Olesen L., Christensen J.H., Højgård A., Granby K., Timm-Heinrich M. (2010) Influence of smoking parameters on the concentration of polycyclic aromatic hydrocarbons (PAHs) in Danish smoked fish. Food Additives & Contaminants: Part A Volume 27, Issue 9; 1294-1305.

EFSA (2008) Polycyclic Aromatic Hydrocarbons in Food. Scientific Opinion of the Panel on Contaminants in the Food Chain. The EFSA Journal 724, 1-114.

European Commission. 2002. Opinion of the Scientific Committee on Food on the risks to human health of polycyclic aromatic hydrocarbons in food expressed on 4 December 2002. Brussels (Belgium): European Commission. Available from http://europa.eu.int/comm/food/fs/fc/scf/index_en.htlm

Falcó G., Domingo J.L., Llobet J.M., Teixidó A., Casas C., Müller L. (2003). Polycyclic aromatic hydrocarbons in foods: human exposure through the diet in Catalonia, Spain. J Food Prot. 66 (12): 2325-2331.

Garcia-Falcon M.S. and Simal-Gandara J. (1999) Determination of benzo[a]pyrene in some Spanish commercial smoked products by HPLC-FL Food Additives & Contaminants 16, Issue 1, 9-14.

Garcia-Falcon M.S. and Simal-Gandara J. (2005). Polycyclic aromatic hydrocarbons in smoke from different woods and their transfer during traditional smoking into chorizo sausages with collagen and tripe casings. Food Additives and Contaminants, 22, 1-8.

Guillén M.D., Sopelana P., Partearroyo M.A. (2000) Polycyclic aromatic hydrocarbons in liquid smoke flavorings obtained from different types of wood: effect of storage in polyethylene flasks on their concentrations. J. Agric. Food Chem. 48, 5083-5087.

Guillén M.D., Palencia G., Ibargoitia M.L., Fresno M., Sopelana P. (2011)

Contamination of cheese by polycyclic aromatic hydrocarbons in traditional smoking. Influence of the position in the smokehouse on the contamination level of smoked cheese. J Dairy Sci. Apr;94(4):1679-90.

IARC International Agency for Research on Cancer. (2010) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Monographs on the evaluation of Carcinogenic Risk to Humans http://monographs.iarc.fr/ENG/Classification

IPCS, 1998. Selected Non-heterocyclic Polycyclic Aromatic Hydrocarbons. Environmental Health Criteria 202. International Programme on Chemical Safety, World Health.

Lay Description of the Linearized Multistage Model(Crump 1984)

Larsson, B.K., Sahlberg, G., Eriksson, A.T. and Busk, L.A. (1983) Polycyclic aromatic hydrocarbons in grilled food. Journal of Agricultural and Food Chemistry 31, 867-873.

Lodovici M., Dolara P., Casalini C., Ciappellano S., Testolin G.(1995). Polycyclic aromatic hydrocarbon contamination in the Italian diet. Food Addit Contam. 12 (5): 703-713.

Nesnow, S., Ross, J.A., Stoner, G.D., Mass, M.J., 1995. Mechanistic linkage between DNA adducts, mutations in oncogenes and tumorigenesis of carcinogenic environmental polycyclic aromatic hydrocarbons in strain A/J mice. Toxicology, 105, 403-413.

Organization, Geneva. Liu G, Niu Z, Van Niekerk D, Xue J, Zheng L. (2008) Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions, analysis, and toxicology. Rev Environ Contam Toxicol. 192:1-28.

Regolamento della Commissione Europea 1881/2006 che definisce i tenori massimi di alcuni contaminanti nei prodotti alimentari. Gazzetta ufficiale dell'Unione europea, L364 del 20/12/2006, 4-8.

Regolamento della Commissione Europea (EU) 835/2011 del 19 Agosto 2011 che modifica il Regolamento (EC) 1881/2006 riguardo ai tenori massimi di idrocarburi policiclici aromatici nei prodotti alimentari. Gazzetta ufficiale dell'Unione europea, L215 del 20/08/2011, 4-8.

Regolamento della Commissione Europea (EU) 836/2011 del 19 Agosto 2011 che modifica il Regolamento (EC) 333/2007 che stabilisce i metodi di campionamento e di analisi per il controllo ufficiale di dei tenori di piombo, cadmio, mercurio, stagno inorganico, 3-MCPD e benzo(a)pirene nei prodotti alimentari. Gazzetta ufficiale dell'Unione europea L215 del 20/08/2011, 9-16.

R.E. Lehr, D.M. Jerina, 1977. Metabolic activations of polycyclic hydrocarbons. Archives of Toxicology, Volume 39, Issue 1-2, pp 1-6.

Riha W.E.; Wendorff W.L.; Rank S. (1992) Benzo(a)pyrene content of smoked and smoke-flavored cheese products sold in Wisconsin. Journal of food protection (USA). 55 (8), 636-638.

Simko P. (2005) Factors affecting elimination of polycyclic aromatic hydrocarbons from smoked meat foods and liquid smoke flavorings. Mol Nutr Food Res. Jul;49(7):637-47.

Suchanovà M., Hajs lovà J., Tomaniovà M., Kocourek V., Babicka L. (2008) Polycyclic aromatic hydrocarbons in smoked cheese J Sci Food Agric 88, Issue 8; 1307–131.

2. Objectives of the thesis project

The aims of this work are:

1) The analysis and the comparison of PAH contamination profiles in smoked buffalo mozzarella produced in Campania, processed with different techniques such as straw, wood, cardboard combustion and liquid smoke.

2) Detection of PAH4, PAH2, Benzo[K]Fluoranthene and dibenzo[a,h]Anthracene in smoked mozzarella.

3) Evaluation of possible migration of these contaminants from surface to core of mozzarella.

3. RESULTS AND DISCUSSION

3.1 Topic/theme

The field of buffalo mozzarella production, a typical DOP product, contributes to increasing of "made in Italy" exports worldwide. In the last 15-20 years buffalo farms and dairy farms were increased, at now there are over 2000 businessmen and 250 dairies (only 128 dairies have DOP certification) and over 20 thousand employees. Today the buffalo assets counts around 250000 heads, involving about 130000 lactating buffaloes, distributed in 1850 farms. The 80% is distributed in the Campania region; the remaining 20% is distributed in south Lazio, Puglia and Molise. In Campania, about 33 thousand tons of DOP buffalo mozzarella are produced in 2008), with a constant average increase in the last decade (Regione Campania, 2013). The consumption trend is positive with an annual increase of about 10%.

Frequently buffalo mozzarella is subjected to a smoking process, for increasing shelf-life and giving cheese aroma and flavour, the obtained product was called "Provola". This process, as before described, could produce toxic and carcinogenic PAHs, that can affect human health.

In guidelines of mozzarella production, smoking process is still not well regulated and, in many cases, is done using empirical techniques and uncontrolled parameters (such as temperature, humidity and ventilation). During the smoking process can be used different materials such as bark of alder, wealth straw and chips of fir or beech but in same dairy farms not legally tools (such as cardboard) are used.

However, the part of smoking mozzarella or provola, that is most exposed to smoke which receiving aroma and flavour, originated during this process, is the outer layer, i.e. the rind. This part could serve as a barrier to the entry of PAHs in the inner mozzarella favouring PAH accumulation on the rind, or it could permitted their migration to the mozzarella core.

For this reason in this study the presence of PAHs in smoked mozzarella and relative consumer exposure were evaluated. It was studied the influence of different smoking techniques and used materials on the formation of PAHs in mozzarella, and the possible their migration from rind to core of the mozzarella, too.

3.2 Materials and methods

3.2.1 Sampling

In this study 50 batches of DOP buffalo mozzarella of Campania (MBC) were collected from a dairy farm located in the Salerno province (Campania Region) and with CE mark. The sampling was carried out during ten months, specifically from June 2012 to March 2013. Every batch consisted of 16 mozzarella, each of them

weighted about 200-250 gr. For each batch, one non smoked mozzarella (blank mozzarella), was collected and analyzed is as. The remaining 15 mozzarella of the batch were transported, immediately after production, to another dairy farm located in the same province to be subjected to smoking treatment. These mozzarella were divided in groups of three, each group was subjected to a smoking process. In particular:

• 3 mozzarella were smoked with the corrugated cardboard

• 3 mozzarella were smoked using liquid smoke

• 3 mozzarella were smoked with straw

• 3 mozzarella were smoked with alder bark

• 3 mozzarella were smoked with chips of fir or beech

For each different technique, exposure time was monitored, in fact, it varied from 2.5 minutes to 15 minutes. Some parameters of the smoking process, as the temperature, the ventilation, and the humidity, could not be monitored because of they were empirically chosen by the employees.

Usually the traditional smoking techniques were carried out in a metal structure as a smoking room (height = 100 cm, circumference = 60 cm), the bottom was put different combustion materials, and on the top wet sack. Mozzarella were suspended with a rope or placed on a metal grid, and exposed to combustion smoke for a period varied from 2.5 to 6 minutes for cardboard (illegal technique)(Figure 9) , from 2 to 15 minutes for wheat straw, alder bark and beech shavings smoke, then dried for a few minutes and immersed in brine. (Figures 10, 11 and 12)

Figure 9- Smoked MBC with corrugated cardboard

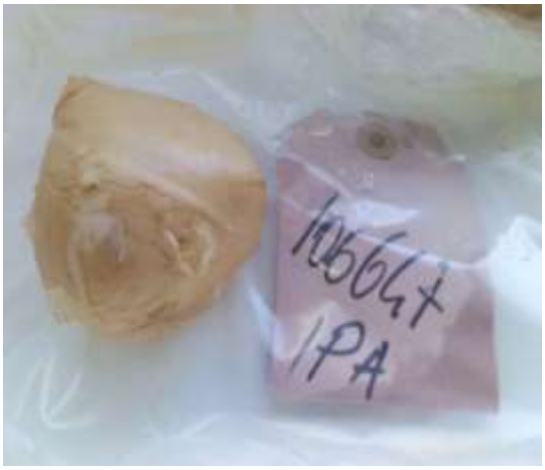
Figure 10- Smoked MBC with wheat straw smoke

Figure 11- Smoked MBC with alder bark smoke

Figure 12- Smoked MBC with fir or beech shavings smoke

The smoking process with "liquid smoke" (Figure 13) consists of a solution in which the samples are immersed for 5 to 10 minutes and then dried at room temperature for no more than 2 minutes.

Figure 13- Smoked MBC with liquid smoke



3.2.2 Analysis and Determination of PAHs

After smoking treatment, mozzarella were transported at the laboratory of the Istituto Zooprofilattico Sperimentale del Mezzogiorno of Portici where they were cataloged, labeled and recorded. The blank mozzarella was homogenized, divided into aliquots and analyzed. (Figure 14)

Figure 14- Not smoked MBC

For each group of the three smoked mozzarella:

• one mozzarella was entirely homogenized, then divided into aliquots and analyzed

• one mozzarella was divided into outer (the rind) and inner (the core) part, which were separately homogenized and analyzed

• one mozzarella was stored in order to probable confirmation of data

3.2.3 Equipment and materials

- Balance sheet (accuracy ± 0.01 g), Blender blades Water bath,Test tubes Falcon polypropylene screw cap 50 mL, Glass tubes with a conical base 10 mL, Balls glass conical bottom 100 mL, Filter paper 150 mm, Vacuum manifold chromatography using SPE, Rotary evaporator, Evaporator under a stream of nitrogen, Silica SPE Columns 3 mL/500 mg VAC, Agitator Vortex, Column chromatography EnviroSep PP 125*3.2 mm, particles from 5 µm (Phenomenex), with pre-C18 column, System HPLC(Acquity-Waters) with fluorimetric detection (FLD)

3.2.4 Chemicals and reagents

HPLC grade acetonitrile (ACN), RPE Potassium hydroxide (KOH), RPE anhydrous Sodium sulfate, Cyclohexane for pesticide analysis and Ethanol (96-98% purity grade) were from Carlo Erba (Milan, Italy). Ultrapure water was in-house produced using a MilliQ Laboratory System (Millipore, Bedford, MA, USA). Sep-Pak® Vac 3cc (500 mg) Silica SPE Cartridges were purchased by Waters (Milan,Italy)

3.2.5 Reference materials

Benzo[a]Pyrene (BaP, 99.6% ± 2.0% analytical purity grade) solution at 10 µg/mL in ACN, Benzo[k]Fluoranthene (BkF, 99.0% ± 2.0% analytical purity grade) solution at 10 µg/mL in ACN, Benzo[b]Fluoranthene (BbF, 99.5% ± 2.0% analytical purity grade) solution at 10 µg/mL in ACN, Benzo[a]Anthracene (BaA, 99.5% ± 2.0% analytical purity grade) solution at 10 µg/mL in ACN, diBenzo[a,h]Anthracene (dBahA, 99.5% ± 2.0% analytical purity grade) solution at 10 µg/mL in ACN and Chrysene (Cry, 99.2% ± 3.0% analytical purity grade) solution at 10 µg/mL in ACN were producted by Dr. Ehrenstorfer GmbH (Augsburg, Germany)

3.3 Procedure

3.3.1 Preparation of intermediate and working solutions

Mix standard solutions at 0.4 – 1.0 – 2.5 – 5.0 – 10.0 – 20.0 ng/mL were prepared daily by dilution in acetonitrile of mix standard solution at 0.1 µg/mL and used to calculate calibration standard curves. The working standard solution should be prepared immediately before use and discarded after use.

A solution of KOH 1 M in ethanol was prepared by dissolution of 5.60±0.01 g of KOH in ethanol to a final volume of 100 mL.

3.3.2 Sample extraction

Mozzarella samples were homogenized by a blade blender, then 2.00±0.01 g were weighed in a 50 mL Falcon tube. In each working session, a spiked sample was prepared by adding 100 µL of mix standard solution at 0.1 µg/mL to 2.00±0,01 g of a blank sample. Each sample was added with 10 mL of KOH 1 M solution in ethanol, then, mixed by vortex for 1 minute and put in thermic bath at 80°C ± 2°C for 2 hours. After cooling at room temperature, 10 mL of ultrapure water and 20 mL of cyclohexane were added to samples, mixed further by vortex for 30 seconds. After centrifugation at 3000 rpm for 5 minutes, supernatant was transferred into a 100 mL flask by passing it through an anhydrous sodium sulphate filter. This step was carried out to times. Filtered organic phases were collected up and reduced to a volume of about 0.1 mL under vacuum using a rotovapor set at 45°C. Residue was dissolved in 2 + 1 mL of ACN and loaded onto Sep-Pak® Silica Cartridge, previously conditioned with 3 mL of ACN, the elute was collected in a glass tube. Further 3 mL of ACN were loaded onto the cartridge and collected in the previous glass tube to ensure complete elution of compounds of interest. Purified extract was then evaporated under nitrogen stream at T≤40°C and residue was dissolved with 1

mL of ACN for HPLC-FLD analysis.

Extracted samples, a reagent blank (ACN), calibration curve standards at 0.4 – 1.0 – 2.5 – 5.0 – 10.0 – 20.0 ng/mL, blank sample utilized for spiked sample and spiked sample itself were analyzed during each working session. For each sample to be analyzed, 300 µL were transferred in vials befitting for HPLC autosampler. Instrumental analyses were performed by means of an Ultra Performance LC system equipped with a fluorescence detector (Waters, Ireland).

PAHs were separated on 5 µm particles 125x3.2 mm Envirosep PP (Phenomenex) column previously conditioned with ultrapure water/acetonitrile 20/80 (v/v) at a flow rate of 0.5 mL/min. Chromatography was performed at a temperature of 25°C ± 2°C using as mobile phases ultrapure water (phase A) and acetonitrile (phase B) at a flow rate of 0.5 mL/min. Each Injection batch was: reagent blank (acetonitrile), blank sample, six mix standard solutions of reference material at different concentrations, spiked sample, a blank and the samples.

Compounds separation was achieved by applying the following linear gradient:

Time (minutes)	0	15	20	25
A %	20	0	0	20
B %	80	100	100	80

Fluorescence detector was set at excitation wavelengths (λ_{ex}) of 294 nm and emission wavelengths (λ_{em}) of 404 nm.

PAHs presence was pointed out by peaks in the chromatogram at the same retention time of those observed in calibration curve standards, with an admitted tolerance of ±2.5%.

3.4 Validation test

A co-chromatography was performed by adding 200 µL of mix standard solution at different concentration 2.5 ng/mL to 200 µL of the sample solution and injecting it. The peak area must be proportional to the amount of injected PAHs and doubling of the chromatographic peak does not have to be observed.

3.5 Criteria and / or requirements for approval / rejection of the test

As established by EU Regulation 836/2011, if spiked sample showed peaks corresponding to PAHs peaks of mix standard solutions and if recoveries for Benzo[a]Pyrene and the other three PAHs (B[a]A, B[b]F and Cry) ranged from 50% to 120%, the test was evaluated as acceptable. Otherwise, test must be repeated.

3.6 Expression of results

PAHs concentrations were evaluated by interpolation with calibration curves. The results were expressed as mg/kg (µg/g).

3.7 Statistical Analysis

To evaluate the influence of smoking time and smoking techniques on ΣPAH contamination levels, their contents in the core, rind and whole smoked mozzarella cheese ready to eat were compared. Besides in order to evaluate if the rind can represent a barrier to PAH migration into the core, the levels found in the rind were compared to values found in the core. To approximate our results as a normal distribution of the data they were log-transfored. All mean results are upper estimates that assume PAHs not detected as present at the limit of detection. Statistical analysis was carried out by paired sample t-test. The level of significance was set at $p<0.05$.

3.8 Dietary Intake

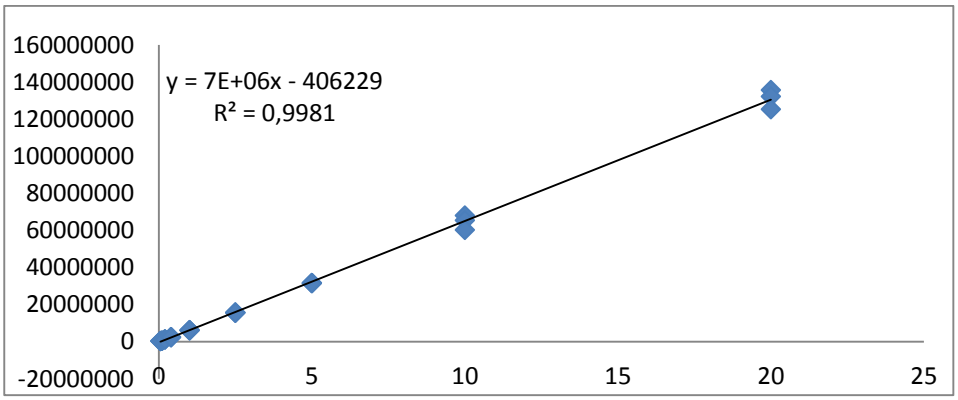
In order to evaluate the daily intakes of BaP, PAH2 and PAH4 through smoked mozzarella cheese ingestion, as suggested by EFSA, the median ingestion of 42 g of cheese was multiplying to median concentrations of BaP, PAH2 and PAH4 found in this study. These values are expressed as ng/day. Then the daily intakes per person were calculated dividing the obtained results by an average body weight (bw) of 60 kg and the results were showed as ng/g/bw/day, in according to EFSA, 2008.

3.9 Method Validation

In developing the method, the performance criteria set out in Commission Regulation EU 836/2011 for the determine analysis of PAHs were evaluated. The concentrations of PAH in the samples was determined by the method of external standard. The linearity of the method was verified in the range of 0.001-0.020 mg/L, corresponding to concentration values between 0.005 and 10 µg/kg. Linear least square regression was applied in order to construct a calibration line showing peak area vs. PAH concentration. The value of r^2 was higher than 0.99 for all six PAHs, thus demonstrating good linearity.

Figure 15 shows the calibration line obtained for BaP injecting three solutions of reference material of each concentration level.

Figure 15 – Calibration line for BaP



The limit of quantification (LOQ = 0.2 µg kg-1) was calculated for each PAH by using a blank sample fortified with a solution of the six PAHs; the limit of detection (LOD) was 0.1 µg kg-1.The precision of the analytical method was determined by

spiking a blank sample with the calibration standard solution at 0.1 µg/L and carrying through the entire extraction and clean-up procedure as for the samples. Precision was evaluated by calculating the coefficient of Horrat for each PAH at a concentration of 5.0 µg kg-1, under conditions of repeatability (Horrat r) and reproducibility (Horrat R). In terms of repeatability, precision was also assessed through the analysis of the blank sample spiked at concentrations of 0.2, 0.5 and 1.0 µg/kg in at least five replicates. Another five replicates for each level were analyzed by a different operator with different lots of reagents and solvents, on different days, to obtain the reproducibility value. Accuracy was then calculated as the mean recovery percentage for all individual PAHs and compared with the interval of acceptability provided by EU Regulation 836/2011: in all cases the recovery values obtained were in the range 50-120%, as required.

Table 5 – Results of recovery tests at levels 2.0 and 5.0 µg / kg of matrix buffalo mozzarella

	RECOVERY TEST	CONC.	REC. %		RECOVERY TEST	CONC.	REC. %
I session	2,0	1,6	77,5		5,0	3,3	66,0
	2,0	1,5	72,5		5,0	4,2	84,0
	2,0	1,5	77,0		5,0	3,3	66,0
	2,0	1,6	77,5		5,0	3,5	70,0
	2,0	1,5	75,5		5,0	3,1	62,0
	2,0	1,5	75,5		5,0	3,4	68,0
MEDIA		*1,52*	*75,9*			*3,467*	*69,3*
DEV STD		*0,04*	*1,9*			*0,383*	*7,7*
RSD, %		*2,51*	*2,5*			*11,05*	*11,0*
II session	2,0	1,6	78,2		5,0	4,2	84,0
	2,0	1,6	78,4		5,0	4,2	84,0
	2,0	1,7	82,9		5,0	4,2	84,0
	2,0	1,5	77,5		5,0	3,8	76,0
	2,0	1,6	78,9		5,0	3,8	76,0
	2,0	1,7	85,2		5,0	3,8	76,0
MEDIA		*1,60*	*80,2*			*3,97*	*79,4*
DEV STD		*0,06*	*3,1*			*0,21*	*4,3*
RSD, %		*3,91*	*3,9*			*5,38*	*5,4*

Specificity was assessed by verifying the absence of interfering peaks at round to 2.5% of the Rt of the individual PAHs in the chromatogram of twenty samples of PAH-free dairy products.

For analytical quality assurance measures in each batch of samples, a procedural blank and a spiked sample were included.

In Figure 16 shows the chromatogram HPLC-FLD obtained for a solution of the reference materials, all six IPA sought. In Figures 17 and 18, also shows the chromatograms, respectively, relative to a sample of mozzarella not smoked and a sample of smoked buffalo mozzarella. As an example chromatogram was shown on the smoked with corrugated cardboard.

Figure 16 - Chromatogram of reference material/standard

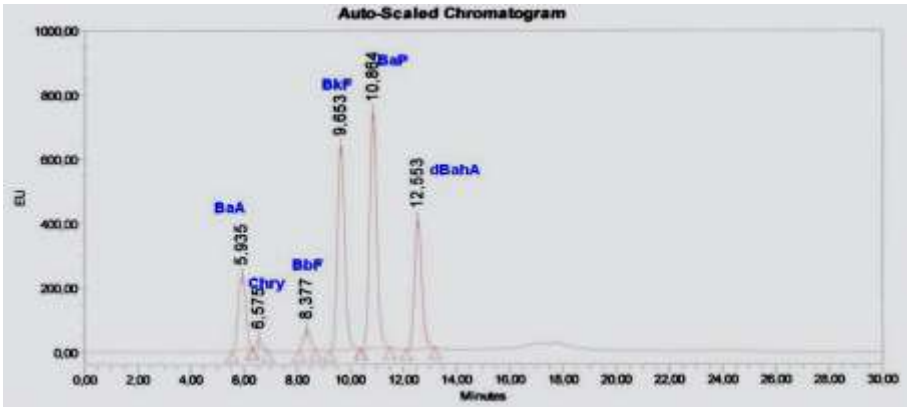


Figure 17-Chromatogram of untreated buffalo mozzarella

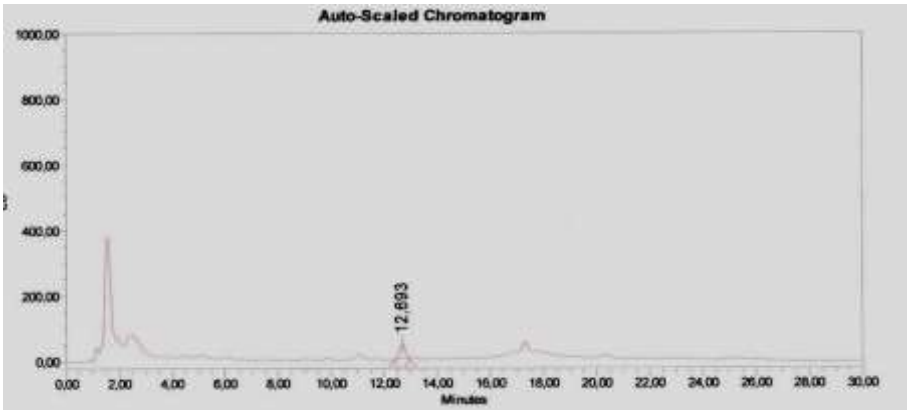
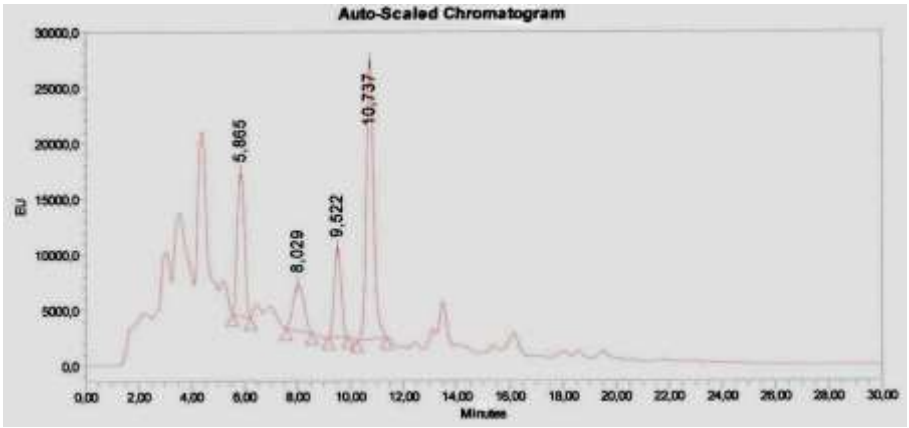


Figure 18 - Chromatogram of smoked buffalo mozzarella (with corrugated cardboard)



Concentrations are expressed as µg kg^{-1} (wet weight) and all results were corrected for recovery, as Commission Regulation EU 836/2011 requires for benzo[a]pyrene and other PAHs. Values below the quantification limit are reported as < LOQ (0.2 µg kg^{-1}).

3.10 Results and discussion

All non smoked mozzarella samples were not contaminated by PAHs; for this reason they were used as blank sample in each working session. (Table 6)

Table.6 Concentrations of PAHs (µg/Kg) in untreated mozzarella

PAH	Mean ± S.D.	Median (Range)
BaA	< 0.2	< 0.2
Cry	< 0.2	< 0.2
BbF	< 0.2	< 0.2
BKF	< 0.2	< 0.2
BaP	< 0.2	< 0.2
DBahA	< 0.2	< 0.2
PAH4	< 0.2	< 0.2

The core of all the samples of smoked mozzarella (Tab. 7) was not contaminated.

By analysis of the rind of mozzarella smoked with corrugated paper were found levels of Benzo[a]Pyrene highest that ranged from 110 to 417.8 µg/kg with a median value of 242.4 µg/kg and PAH4 showed a median value of 1039.4 µg/kg and levels ranged from 957.2 µg/kg to 1892 µg/kg (Table 8).

For liquid smoked samples, there were no appreciable levels of detected PAHs in the rind. Among traditional techniques alder wood combustion showed for the rind highest Benzo[a]Pyrene levels (range 5.1 µg/kg - 54.0 µg/kg, median 32.1 µg/kg) followed by Chips of fir or beech (range 1.5 µg/kg - 44.3 µg/kg, median 22.8 µg/kg) and then by wheat straw (range 0.2 µg/kg - 28.2 µg/kg, median 8.6 µg/kg). About PAH4 similar trend was found, in fact alder wood showed highest values in a range of 25.6 µg/kg - 176.0 µg/kg (median of 94.8 µg/kg), followed by Chips of fir or beech with a range of 4.9 µg/kg - 146.3 µg/kg (median 71.7 µg/kg) and by wheat straw with a levels that ranged from 0.2 µg/kg to 248.7 µg/kg (median 64.6 µg/kg) (Table 8).

In table 9 the results about PAH levels found in whole products were showed. In particular, about the illegal use of not authorized material, according to those listed in Regulation (EC) No.2065/2003, such as cardboard, levels of Benzo[a]Pyrene ranged from 3.1 to 25.8 µg/kg with a median of 8.3 µg/kg and the levels PAH4 varied from 12.2 to 130 µg/kg with a median value of 43.4 µg/kg (Table 9).

For liquid smoked samples, PAH levels were always not detectable.

The results obtained for smoking trough alder wood and Chips of fir or beech were quite similar both for BaP and PAH4. In fact for Bap, the medians were 2.8 µg/kg

and 1.2 µg/kg, respectively; while for PAH4 they were 9.3 µg/kg and 10.8 µg/kg, respectively.

Instead, for wheat straw in the whole product, the evaluated concentration of Benzo[a]Pyrene ranged from “not detectable” 4.4 µg/kg (Table 9), with a median value of 0.4 µg/kg and PAH4 median was 2.8 µg/kg (range 0.2 µg/kg - 26.2 µg/kg) (Table 9).

Tab.7 Contents of PAHs (μg kg^{-1} w.w.) in core cheese obtained with different smoking techniques and smoke materials

PAH	Smoking techniques and materials smoke									
	Corrugated cardboard		Liquid smoke		Bark of alder		Weath straw		Chips of fir or beech	
	Mean ± S.D.	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*
BaA	<0.2	< 0.2 *(<0.2-12.4)*	<0.2	<0.2	<0.2	<0.2	<0.2	< 0.2 *(<0.2-1.1)*	<0.2	< 0.2 *(<0.2-0.5)*
Cry	<0.2	< 0.2 *(<0.2-5.5)*	<0.2	<0.2	<0.2	<0.2	<0.2	< 0.2 *(<0.2-1.3)*	<0.2	< 0.2
BbF	<0.2	< 0.2 *(<0.2-17.2)*	<0.2	<0.2	<0.2	<0.2	<0.2	< 0.2 *(<0.2-0.6)*	<0.2	< 0.2 *(<0.2-0.5)*
BKF	<0.2	*< 0.2 (<0.2-3.0)*	<0.2	<0.2	<0.2	<0.2	<0.2	*< 0.2 (<0.2-0.2)*	<0.2	*< 0.2*
BaP	<0.2	*< 0.2 (<0.2-8.3)*	<0.2	<0.2	<0.2	<0.2	<0.2	*< 0.2 (<0.2-2.5)*	<0.2	*< 0.2*
DBahA	<0.2	< 0.2	<0.2	<0.2	<0.2	<0.2	<0.2	*< 0.2 (<0.2-0.6)*	<0.2	*< 0.2*
PAH4	<0.2	*< 0.2 (<0.2-43.4)*	<0.2	<0.2	<0.2	<0.2	<0.2	*< 0.2 (<0.2-3.6)*	<0.2	*< 0.2 (<0.2-1.0)*

Tab.8 Contents of PAHs (µg kg^{-1} w.w.) in rind obtained with different smoking techniques and smoke materials

PAH	Smoking techniques and materials smoke									
	Corrugated cardboard		Liquid smoke		Bark of alder		Wheat straw		Chips of fir or beech	
	Mean ± S.D.	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*
BaA	498.7± 172.6	566.0 *(270-702.7)*	<0.2	<0.2	39.5±29.6	37.5 *(9.3-73.8)*	24.5±33.4	8.9 *(<0.2-131.4)*	37.1± 39.9	34.4 *(2.2-77.3)*
Cry	177.8± 95.3	175.6 *(<0.2-296.6)*	<0.2	<0.2	20.7±22.4	18.1 *(<0.2-46.7)*	10.3±13.7	6.9 *(<0.2-41.9)*	7.3±12.2	1.8 *(<0.2-25.5)*
BbF	410.6±161.7	481.9 *(<0.2-509.9)*	<0.2	<0.2	8.9±1.9	8.1 *(<0.2-11.2)*	2.4± 28.2	11.0 *(<0.2-84.5)*	6.3±7.5	5.5 *(<0.2-14.5)*
BKF	91.5±32.7	85.3 *(61.0-138.0)*	<0.2	<0.2	11.1±8.5	12.1 *(1.5 -18.6)*	2.4±2.7	2.3 *(<0.2-8.8)*	8.1±8.5	8.2 *(0.6-15.6)*
BaP	247.8±117.0	242.4 *(110-417.8)*	<0.2	<0.2	30.8±23.8	32.1 *(5.1-54.0)*	11.0±9.1	8.6 *(<0.2-28.2)*	22.8± 24.2	22.8 (1.5-44.3)
DBahA	< 0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.6±1.5	<0.2 *(<0.2-6.5)*	0.5±0.6	0.4 *(<0.2-1.2)*
PAH4	1217.3±385.3	1039.4 *(957.2-1892)*	<0.2	<0.2	97.8±75.3	94.8 *(25.6-176.0)*	72.5±74.7	64.6 *(<0.2-248.7)*	73.6±70.2	71.7 *(4.9-146.3)*

Tab.9 Contents of PAHs (µg kg^{-1} w.w.) in whole cheese obtained with different smoking techniques and smoke materials

PAH	Smoking techniques and materials smoke									
	Corrugated cardboard		Liquid smoke		Bark of alder		Weath straw		Chips of fir or beech	
	Mean ± S.D.	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*	Mean ± S.D	Median (*Range)*
BaA	20.2± 14.6	12.4 *(7.3-41.1)*	< 0.2	< 0.2	3.8± 1.2	4.0 *(2.1-5.1)*	1.5± 2.3	0.9 *(<0.2-4.3)*	3.1± 4.2	1.5 *(<0.2-9.3)*
Cry	5.1± 4.3	5.5 *(<0.2-10.9)*	< 0.2	< 0.2	0.9± 1.2	0.4 *(<0.2-2.7)*	0.8± 1.3	<0.2 *(<0.2-3.8)*	1.7± 2.0	1.7 *(<0.2-3.7)*
BbF	26.2± 21.2	20.6 *(<0.2-55.6)*	< 0.2	< 0.2	1.8± 3.4	0.1 *(<0.2-6.9)*	1.7± 2.6	<0.2 *(<0.2-8.0)*	4.1± 6.9	1.0 *(<0.2-14.5)*
BKF	3.9± 2.9	3.0 *(1.3-8.9)*	< 0.2	< 0.2	1.2± 0.2	1.2 *(0.9-1.5)*	0.4± 0.5	0.2 *(<0.2-1.5)*	0.7± 1.0	0.4 *(<0.2-2.2)*
BaP	11.3± 8.7	8.3 *(3.1-25.8)*	< 0.2	< 0.2	2.9± 1.0	2.8 *(1.8-4.2)*	0.9± 1.2	0.4 *(<0.2-4.4)*	2.1± 2.8	1.2 (<0.2-6.1)
DBahA	< 0.2	<0.2 *(<0.2-4.6)*	< 0.2	< 0.2	0.2± 0.3	<0.2 *(<0.2-0.6)*	0.2±0.2	0.3 *(<0.2-0.8)*	0.1±0.1	<0.2 *(<0.2-0.2)*
PAH4	63.0±47.0	43.4 *(12.2-130.0)*	< 0.2	< 0.2	9.4±4.8	9.3 (3.9-15.2)	5.0±7.3	2.8 (<0.2-26.2)	11.1±12.3	10.8 (<0.2-22.8)

These results could be explained if it is considered that the combustion of cardboard always produces a pyrolysis and a pyrosynthesis originating PAHs, whose production is increased if it occurs in oxygen lack. Therefore, in presence of these illegal material such as cardboard or coloured or pesticide-coated wood, there is an increase in quantity of produced PAHs. In our case, the corrugated cardboard was made from two sheets of smooth cardboard with a corrugated tape, tied together by adhesive (starch often used in suspension with caustic soda or adding bioacids). (Carlo P., 2000)

Despite the European legislation does not set limits for dairy products but for other foodstuffs that ranged from 1 to 5 µg/Kg wet weight (ww) for BaP and from 10 to 30 µg/Kg for PAH4, respectively. Our median, about whole mozzarella, were higher than these limits for BaP in samples smoked with cardboard and Chips of fir or beech while for PAH4 only samples smoked with cardboard.

A comparison among literature data was carried out. In particular our results were always lower than Anastasio et al. (2004) for BaP levels in the core of smoked buffalo mozzarella cheeses obtained with different techniques. Besides about the rind, the values of our study were higher than Anastasio research except for liquid smoke. It was tried a comparison between our results concerning the whole product and results obtained by Anastasio et al. but related to slice. So our values were lower than this study for straw and liquid smoke, while higher than Anastasio' experiment for cardboard and wood shaving.

Trying a comparison with data of a study of Guillen et al. (2006), that evaluated the levels of different PAHs in rind of cheeses smoked with Almond shells or Dry prickly pear, our BaA, BbF and BaP results obtained with different smoking techniques were higher than this study except for liquid smoke.

Naccari et al.(2008) evaluated BaA and BaP values in "provola" obtained with two different process, one involved liquid smoke and another the wood combustion. Our BaA and BaP levels were always lower than Naccari' study for liquid smoke, as well as values found in core of mozzarella smoked with wood combustion. However our results about rind and whole products smoked with wood combustion were always higher than Naccari et al. (2008) levels.

In the next figures medians levels of BaP and PAH4 were showed for different techniques and related to core, rind and whole foducts (Figures 19,20,21).

Figure 19 - *Levels of BaP and PAH4 contamination in the core of smoked mozzarella, expressed as median.*

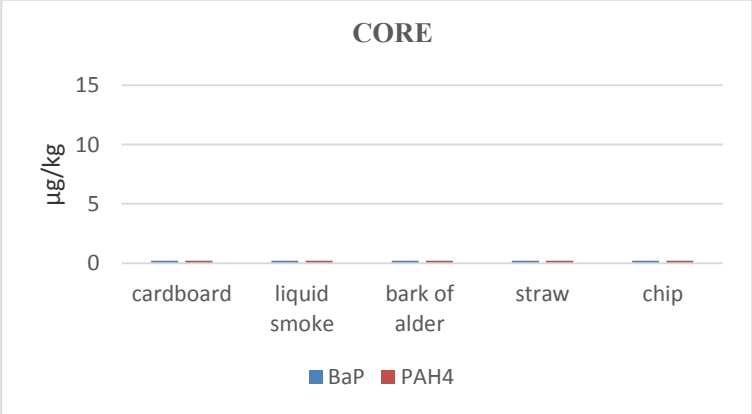


Figure 20 - *Levels of BaP and PAH4 contamination in the rind of smoked mozzarella, expressed as median.*

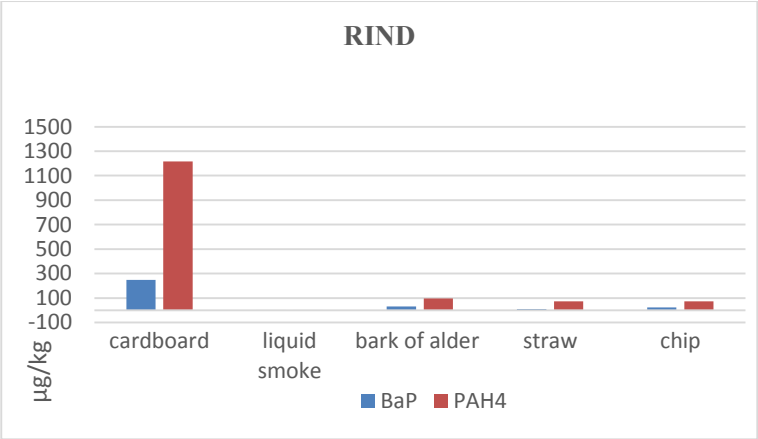
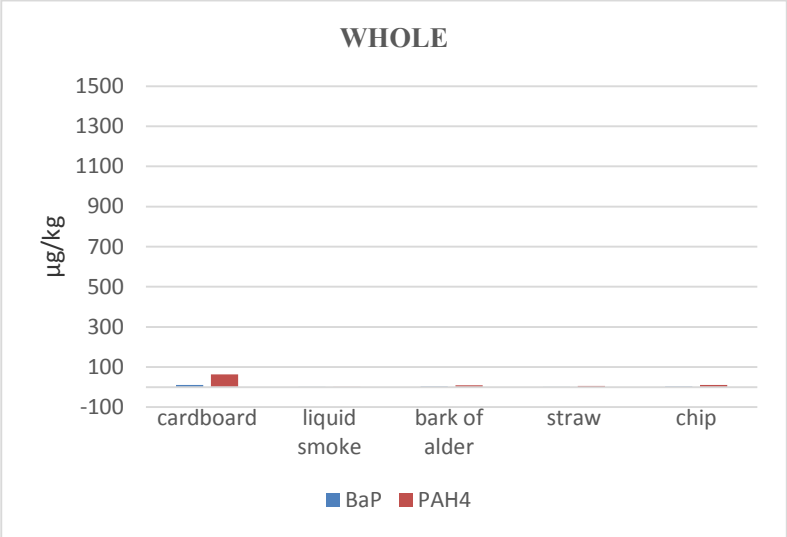


Figure 21 - *Levels of BaP and PAH4 contamination in the whole smoked mozzarella, expressed as median.*



In Figures 22, 23, 24, 25 and 26, it is shown trend of six detected PAHs and PAH4 in rind, core and whole smoked mozzarella with different techniques. It is noteworthy to underline that it was even necessary to reduce 10 times the maximum value of the ordinate axis to make visible histogram of results obtained from samples (core, rind and whole product) treated with liquid smoke or burning fir bark, straw and shavings.

Figure 22- Outline of PAH contamination in mozzarella smoked with corrugated cardboard

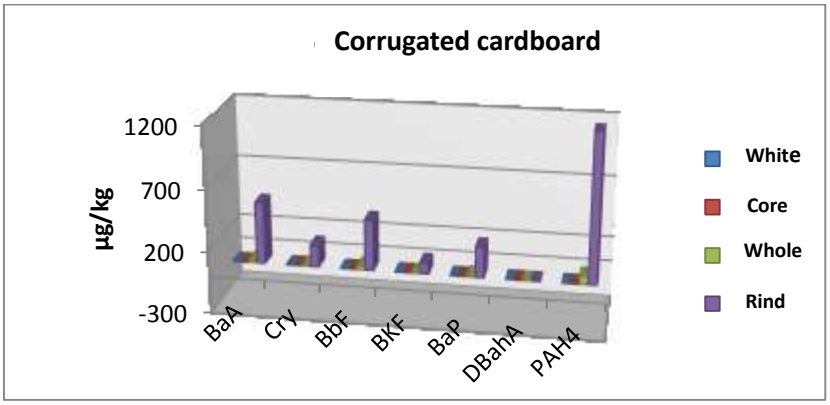


Figure 23 - Outline of PAH contamination in mozzarella smoked with liquid smoke

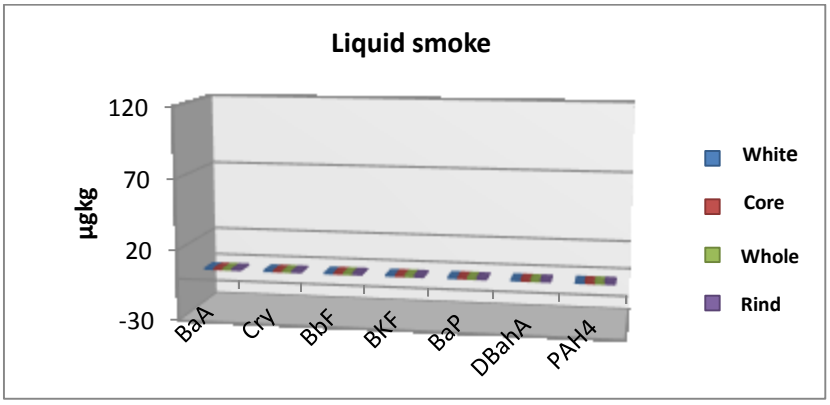


Figure 24 - Outline of PAH contamination in mozzarella smoked with alder bark

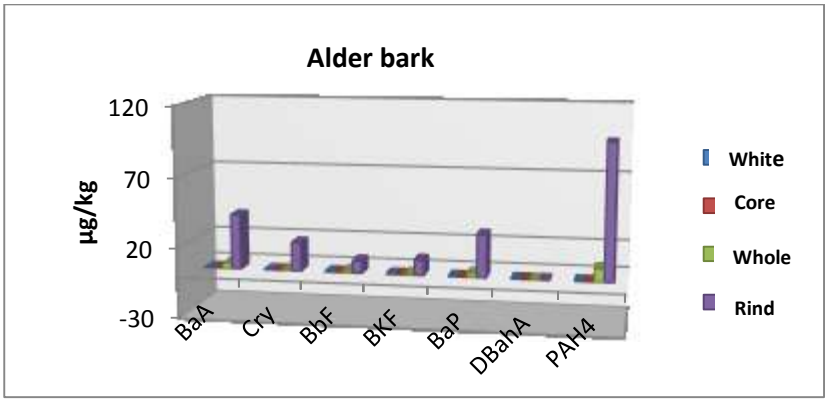


Figure 25 - Outline of PAH contamination in mozzarella smoked with straw

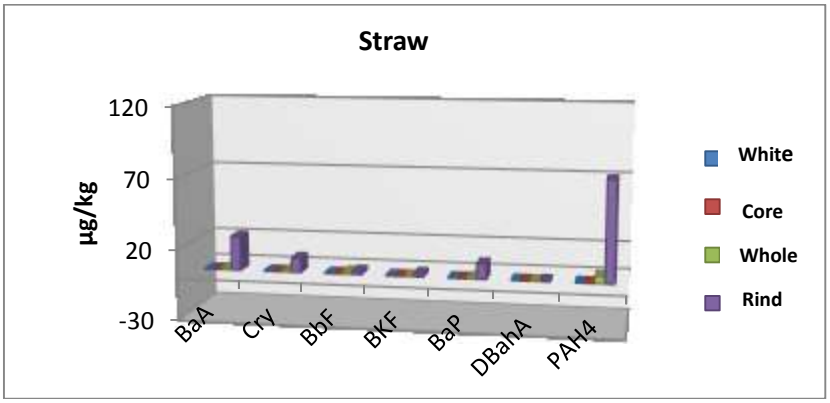
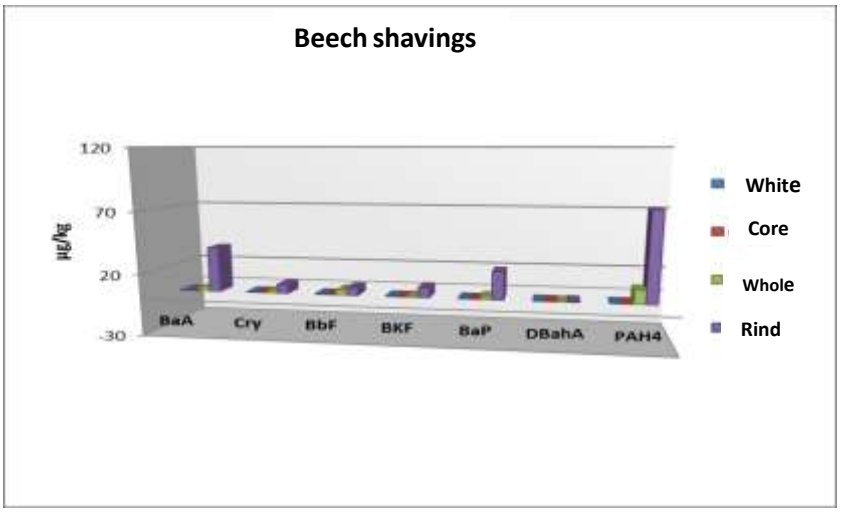


Figure 26 - Outline of PAH contamination in mozzarella smoked with beech shavings



3.11 Statistical Analysis

About the evaluation of incidence of smoking time on PAH formation, statistical analysis was carried out and conventionally the results were grouped considering a smoking time ≤ 4 and > 4 minutes. No statistical differences were found comparing, for each technique, core, rind and whole products, probable because of the PAH formation occurs during the first minutes of smoking process.

The statistical evaluation was carried out among rind and whole smoked mozzarella and rind and core and significant differences were found in both comparisons ($p = 0.000$ rind vs whole product and $p = 0.003$ rind vs core). These results demonstrated that the rind can be considered as a barrier where PAH, after their formation, can remain and so they can not migrate from the rind to the core.

3.12 Human exposure assessment

Daily was calculated as above described so the median found BaP, PAH2 and PAH4 levels were multiplying to median ingestion of 42 g (EFSA, 2008). The obtained results about BaP, PAH2 and PAH4 intakes due to ingestion of smoked mozzarella samples subjected to different smoking techniques were showed (Tab. 10). BaP values ranged from 0 (liquid smoke) to 348.6 ng/day (cardboard), PAH2 from 0 (liquid smoke) to 579.6 ng/day (cardboard) and PAH4 0 (liquid smoke) to 1822.8 ng/day (cardboard). Besides considering all samples the found intakes were: 42 ng/day (BaP), 56.7 ng/day (PAH2), and 163.8 ng/day (PAH4).

In the second step the intakes for person were evaluated as daily intake for an adult of 60 Kg. Values obtained ranged from 0 (liquid smoke) to 5.8 ng/day/Kg bw (cardboard), PAH2 from 0 (liquid smoke) to 9.7 ng/day/Kg bw (cardboard) and PAH4 0 (liquid smoke) to 30.8 ng/day/Kg bw (cardboard). For all samples the intakes were 0.7 ng/day/Kg bw for BaP, 0.9 ng/day/Kg bw for PAH2 and 2.7 ng/day/Kg bw for PAH4 (Tab.11).

Table 10- Median of daily intake (ng/day) about BaP, PAH2 and PAH4 due the ingestion of whole mozzarella cheese.

MATERIALS	**BaP** ng/day	**PAH2** ng/day	**PAH4** ng/day
STRAW	**16.8**	**33.6**	**117.6**
BARK OF ALDER	**117.6**	**138.6**	**390.6**
LIQUID SMOKE	**0**	**0**	**0**
CARDBOARD	**348.6**	**579.6**	**1822.8**
CHIPS	**50.4**	**128.1**	**453.6**
TOTAL	**42**	**56.7**	**163.8**

Table 11- Median of daily intake for person (ng/day/Kg body weight) about BaP, PAH2 and PAH4 due the ingestion of whole mozzarella cheese of an adult of 60 Kg.

MATERIALS	**BaP** ng kg $^{-1}$ bw day $^{-1}$	**PAH2** ng kg $^{-1}$ bw day $^{-1}$	**PAH4** ng kg $^{-1}$ bw day $^{-1}$
STRAW	**0.3**	**0.6**	**2.0**
BARK OF ALDER	**2.0**	**2.3**	**6.5**
LIQUID SMOKE	**0**	**0**	**0**
CARDBOARD	**5.8**	**9.7**	**30.8**
CHIPS	**0.8**	**2.1**	**7.6**
TOTAL	**0.7**	**0.9**	**2.7**

Comparing obtained values with daily intakes, considering only cheese, reported by EFSA, (0.1 ng kg $^{-1}$ bw day $^{-1}$ for BaP, for PAH2 0.2 ng kg $^{-1}$ bw day $^{-1}$and for PAH4 0.3 ng kg $^{-1}$ bw day $^{-1}$) , our data are always higher than daily intake and intake for person, except for liquid smoke. This suggests that mozzarella subjected to treatment with liquid smoke is preferable to the those smoked with traditional procedures.

3.13 Conclusions

In conclusion, this study examined the levels of BaP, BaA, Chry, BbF, BkF and dBahA in smoked mozzarella produced in Campania. The results indicated that the type of smoking process (type of wood or other smoke-generating materials) strongly influences formation of polycyclic aromatic hydrocarbons.
It was found that all samples of smoked mozzarella with different techniques shown that levels of PAH were highest in the rind and usually equal to LOD in the core, as demonstrated by statistical analysis, hence this suggested that no appreciable migration occurred. Therefore PAH concentrations found in the whole mozzarella are mainly due to the contamination of rind.

Considering the diffuse consumption of smoked cheese such as provola and the high levels of contamination detected during this study, it seems necessary to establish maximum levels for BaP and PAH4 in smoked cheese too. Polycyclic aromatic hydrocarbon (PAH) concentrations in smoked cheese can represent a relevant source of these compounds and so reach risks for the consumer, above all when the smoking process is carried out under uncontrolled conditions. In fact during this study it was found that the use of traditional techniques that envolved the use of permitted materials such as straw or wood reduce the PAH levels forming during the cardboard combustion.

Moreover, to improve food safety, the use of commercial liquid smoke is preferable to traditional smoking procedures.
Finally, the different patterns of contamination found in mozzarella cheese smoked by means of different techniques could be exploited in order to detect the use of unauthorized procedures (type of wood or other smoke-generating materials) in cheese production.

3.14 References

Anastasio A., Mercogliano R., Vollano L., Pepe T. and Cortesi M.L.(2004). Levels of Benzo[a]pyrene (BaP) in "Mozzarella di Bufala Campana" Cheese Smoked According to Different Procedures: *J. Agric. Food Chem.* **2004,** *52,* 4452□4455

Di Carlo P. (2000) La produzione del cartone ondulato, Relazione finale:7° Corso di tecnologia per tecnici cartari.

EFSA, 2008

Guille´ M.D.,1 Palencia G., Sopelana P., and Ibargoitia M.L.(2006). Occurrence of Polycyclic Aromatic Hydrocarbons in Artisanal Palmero Cheese Smoked with Two Types of Vegetable Matter: *J. Dairy Sci. 90:2717–2725*

Naccari C., Cristani M., Licata P., Giofré F., Trombetta D. (2008) Levels of benzo[a]pyrene and benzo[a]anthracene in smoked "Provola" cheese from Calabria (Italy) Food Additives and Contaminants: *Part B 07; 1, 78-84*.

Regolamento della Commissione Europea (EU) 836/2011 del 19 Agosto 2011 che modifica il Regolamento (EC) 333/2007 che stabilisce i metodi di campionamento e di analisi per il controllo ufficiale di dei tenori di piombo, cadmio, mercurio, stagno inorganico, 3-MCPD e benzo(a)pirene nei prodotti alimentari. Gazzetta ufficiale dell'Unione europea L215 del 20/08/2011, 9-16.

Regolamento della Commissione Europea (EU) 2065/2003 del 10 Novembre 2003 "on smoke flavourings used or intended for use in or on foods"

Regione Campania-Assessorato Agricoltura: Mozzarella di bufala Campana: www.agricoltura.regione.campania.it/tipici/mozzarel-new.html

Gallo P., La Nucara R., Salini M., Hauber T., De Crescenzo M., Guadagnuolo G., Rossini C., **Urbani V**., Maglio P., Bianco R., Guarino A., Serpe L. (2012). Valutazione del rischio per la presenza di contaminanti chimici nel latte di bufala della Campania: XIV congresso nazionale S.I.Di.L.V. Sorrento

Urbani V., Esposito M., Guadagnuolo G., Serpe F.P., Marigliano L., Maddaluno F. (2012). Validation of a method for detection and quantification of polycyclic aromatic hydrocarbons in food. Emirates Journal of Food and Agriculture, ISSN: 2079-052X

Esposito M., **Urbani V**., Marigliano L., Serpe F.P., Marotta M.P., De Nicola C. (2013). Impiego degli idrocarburi Policiclici aromatici quali marker della contaminazione in mozzarelle di bufala affumicate. CISETA XI congresso Italiano di Scienze e Tecnologia degli Alimenti 2013, Fiera Milano

Esposito M., **Urbani V**., Marigliano L., Seccia G., Casamassima F., Gesualdo G., Mambelli P., Nardelli V. (2013). Determinazione di Idrocarburi Policiclici Aromatici in molluschi e pesci prelevati nell'area marina di Taranto. XV Congresso Nazionale S.I.Di.L.V.-Monreale(Pa)

Gallo P., Salini M., Rossini C., **Urbani V**., Serpe L. (2014). New test methods for determination of mycotoxins and heavy metals in vegetables. In: 3rd EAVLD Congress, Pisa

Gallo P., **Urbani V.**, Guadagnuolo G., Fabbrocino S., Serpe L. (2014). Exploring occurrence of coccidiostats and ionophores below tolerable cross contamination levels by HPLC-DAD and ION TRAP LC/ESI-MS/MS methods. In: 7th VDRA Ghent, Belgium