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Determination of High Molecular Mass Polycyclic Aromatic Hydrocarbons in a Typical Italian Smoked Cheese by HPLC-FL

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High-performance liquid chromatography with fluorescence detection was used for the analysis of polycyclic aromatic hydrocarbons (PAHs), in "Diavoletto" smoked cheese. Such cheese is typically produced in the Sorrento peninsula, and it is smoked commonly with different materials of vegetable origin. The importance of the smoking generation material is proven by the attention that the EU is paying in indicating the list of wood that may be used to produce smoking flavor agents. The PAHs considered are classified as "probable human carcinogens" by the U.S. Environmental Protection Agency for sufficient data from animal bioassays. The smoked samples contained high molecular mass PAHs with different levels ranging from 0.12 to 6.21 μg/kg. The determination was carried out also on liquid smoking flavor agents, smoke-flavored cheese, and nonsmoked cheese to measure the level of contamination before the treatment.

KEYWORDS: Smoked cheese; polycyclic aromatic hydrocarbons; high-performance liquid chromatography

INTRODUCTION

The polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment. Combustion of organic matter at high temperature and relatively low oxygen produces small molecular fragments, mostly free radicals, which recombine to form low molecular mass PAHs (two and three rings) as the temperature drops. These latter undergo further synthesis to produce high molecular mass (HMM) (4−6 rings) PAHs (**Figure 1**) (*1*).

PAHs can be generated by natural combustion such as forest fires and volcanic eruptions or by human activities such as industry, heating, waste incineration, and traffic (*1*).

PAHs are compounds of extreme importance for public health, in particular the HMM PAHs. In fact, the mixtures of this HMM PAHs have been associated with human cancer, as reported in Integrated Risk Information System of the U. S. Environmental Protection Agency (EPA) for sufficient data from animal bioassays. This database supplies updated information about tumor sites and intake levels of PAHs when available (*2*).

Some alimentary processes such as grilling, roasting, toasting, and smoking can contribute to PAH formation. The traditional processes of smoking, in which the smoke comes in direct contact with food, can cause the dangerous PAH contamination, if carried out in noncontrolled conditions. Temperature, humidity, type of wood, oxygen concentration during smoke generation, and type of smoke generators can influence PAH production (*3*). For instance, a high content of moisture in wood decreases the combustion efficiency increasing the smoke formation; wood with a low moisture content burns faster, and it can cause incomplete combustion: the optimal moisture content to minimize the particulate emission is between 20 and 30% (*4*).

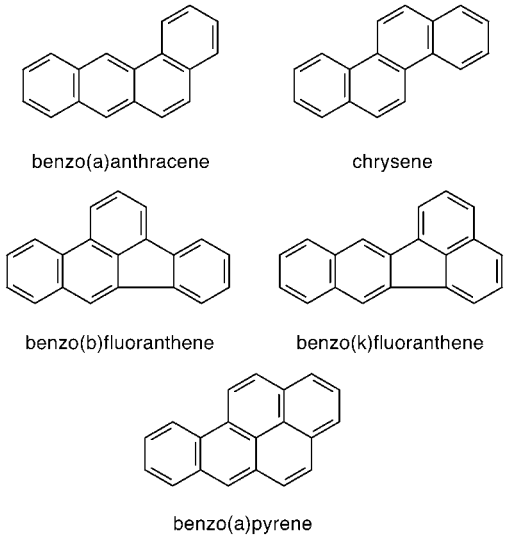

Figure 1. Molecular structure of HMM PAHs found in smoked cheese.

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PAHs are not source specific, since they are found in all biomass combustion emission (*4*); nevertheless, some quantitative considerations related to the type of wood are possible; softwood species (conifers), like pines and firs, are prolific resin producers, which can lead to the production of HMM PAHs such as benzo[*a*]anthracene (BaA), chrysene (Ch), benzo[*a*]-pyrene (BaP) (*1*), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), and indeno(1,2,3-cd)pyrene (IP) (*4*). Conversely, smoke produced by wood of deciduous trees shows low concentrations of Ch, BaA, and BaP (*5*). BaP and Ch are also present in smoke produced by graminaceous (*1*).

To control PAH contamination, the use of smoking flavor agents (SFAs) is becoming frequent to aromatize food products. These agents are also preferred for a better taste reproducibility. SFAs are produced from condensed smoke. Because of the purification process, the use of SFAs is generally considered to be of less health concern than the traditional smoking process. However, these aromas are not completely free from PAH contamination (*6*).

The EU has only set the limit of BaP in flavored foodstuffs at 0.03 μg/kg (*7*). The European Commission announced in the White Paper on Food Safety a proposal regarding a regulation on SFAs used or intended for use in or on foods, confirming the maximum contents in these aromas of BaP and BaA at 10 and 20 μg/kg, respectively (*8*). Source materials for the production of SFAs are also considered; only certain types of untreated wood are allowed (e.g., maple tree, birch, hornbeam, hickory, chestnut tree, eucalyptus, beech, ash, walnut tree, apple, mesquite wood, cherry tree, oak, buckthorn, locust tree, and elm) (*8*). At the present time, no such legislation exists regarding BaP and the other PAHs in smoked foods. The EU is planning to set the limit in smoked food only for BaP at 1 μg/kg as decided in some European countries (*9*).

The measures taken to limit the level of PAHs in foodstuff are focused on BaP (*7*, *10*), used as an indicator of their presence; only the German Society for Fat Science has considered the total HMM PAHs contamination, fixing their limit to 5 μg/kg (*11*).

The intake of BaP from 200 food items was used as a marker of dietary exposure to PAHs (*12*), and a recent review updates PAHs determination in smoked meat and smoke flavoring food additives (*13*).

The present investigation has been carried out on a smoked cheese called "Diavoletto", typical of the Sorrento peninsula. We have studied the following PAHs classified as "B2-probable human carcinogens" from the EPA: BaA, Ch, BbF, BkF, BaP, dibenzo(a,h)anthracene (DBahA), and IP. As reported in IRIS EPA (*2*), the mixtures of this HMM PAH have been associated with human cancer.

The data obtained in cheese submitted to different smoking generation conditions were compared with those obtained in smoke-flavored cheese and liquid smoke flavoring. The PAHs were also determined in cheese before the smoking process.

MATERIALS AND METHODS

Samples. Diavoletto is a fresh small "caciocavallo" cooked paste cheese, stuffed with dried olives, extravirgin olive oil, and chili pepper. Its weight is approximately 50−100 g (32 g of fat and 65 g of dry matter per 100 g of cheese).

Sixteen samples of smoked cheese treated with different wood materials (see **Tables 2** and **3**) were collected directly from one cheese factory with semiindustrial typology (cheese factory A) and another with traditional typology (cheese factory B). In the first case, the smoke was generated in a separate combustion chamber and it was carried in contact with the cheese; while in the traditional procedure, carried on in a wood barrel, the smoke comes in direct contact with the cheese at 40 °C for a maximum of 10 min.

We asked each factory to reproduce their usual smoking procedures. An experimental smoking with cardboard was carried out, to verify the effect of this material as an example of treated vegetable material.

From the same sources were also collected as follows: two samples of liquid SFA, two samples of smoke-flavored cheese, and two samples of cheese before treatment. In this kind of cheese, the rind is edible too; therefore, both rind and paste were analyzed together. All of the analyses were conducted in triplicate.

Reagents. Solvents and reagents were of analytical reagent or high-performance liquid chromatography (HPLC) grade. All of the analytical grade solvents were submitted to further distillation to reduce the PAHs external contamination. Solid phase extraction (SPE) was performed with Isolute (Silica 500 mg/3.0 mL) cartridges obtained from International Sorbent Technology Ltd. (Hengoed, U.K.). PAHs standard mixture TCL PAH Mix (Supelco, Bellefonte, PA) was used.

Apparatus. All analyses were carried out using a Varian 9012 ternary liquid chromatograph equipped with a 20 μL loop, combined with a Varian 9070 fluorescence detector. A Supelcosil LC-PAH 5 μm column (250 mm × 4.6 mm i.d.) (Supelco) was used.

Extraction and Cleanup from Cheese. Ten grams of cheese after homogenization was submitted to lipid extraction according to Christie (*14*). The lipid fraction, after solvent evaporation, was dissolved in 30 mL of cyclohexane and extracted by partitioning with dimethylformamide (DMF)/water (9:1). The DMF/water phase was diluted with 30 mL of water and then submitted to a new extraction with 30 mL of cyclohexane. After the extracted solution was dried over anhydrous sodium sulfate and the solvent was evaporated off, the hydrocarbon fraction was dissolved in 3 mL of *n*-pentane and loaded on a SPE cartridge. The PAH fraction was eluted with 4 mL of dichloromethane/*n*-pentane (2:8) after washing with 1 mL of *n*-pentane. The eluate was concentrated to dryness and redissolved in 200 μL of acetonitrile.

Extraction and Cleanup from Liquid SFA. Ten grams of liquid SFA were extracted with 40 mL of cyclohexane. After it was stirred for 30 min, the mixture was allowed to separate in two phases in a separatory funnel. The lower water phase was reextracted with 30 mL of cyclohexane. The two cyclohexane phases were collected together and concentrated up to 30 mL by a rotary evaporator. The solution obtained was then submitted to the partition and cleanup steps above-described for cheese.

Chromatographic Method. Twenty microliters of acetonitrile solutions were injected into the HPLC system. The gradient elution program used consisted of 60% water and 40% acetonitrile for 5 min, programmed to 100% acetonitrile in 25 min at a flow rate of 1.5 mL/min (*15*). To detect the different PAHs, the programmed conditions of excitation (Ex) and emission (Em) were chosen as follows: time 0−17.6 min, Ex = 224 nm, Em = 320 nm; time 17.6−24 min, Ex = 252 nm, Em = 400 nm; time 24−45 min, Ex = 268 nm, Em = 398 nm (see **Figure 2**).

Quantification of PAHs. The linearity and the quantification of the instrumental response were checked by injecting the PAH standard mixture prepared in five different concentrations ranging from 0.6 up to 200 μg/L. The calibration lines for each PAH were obtained using the linear least squares regression procedure ($n = 3$) of the peak area vs concentration (see **Table 1**).

RESULTS AND DISCUSSION

Recovery, Linearity, and Quantification Limits. Table 1 illustrates the recovery percentages, the linearity, and the limits of quantification (LOQ) of HMM PAHs in cheese. The conversion of the quantification limits of each HMM PAH in the acetonitrile solutions (μg/L) to μg per kg of cheese results from the injection volume and the mass of the sample analyzed.

Smoked Cheese. The total HMM PAHs concentration in smoked cheese analyzed ranged from 0.12 to 6.21 ppb. No quantifiable amounts of DBahA and IP were found. **Tables 2** and **3** show the PAH content determined in cheese submitted to different smoking generation materials. No qualitative dif-

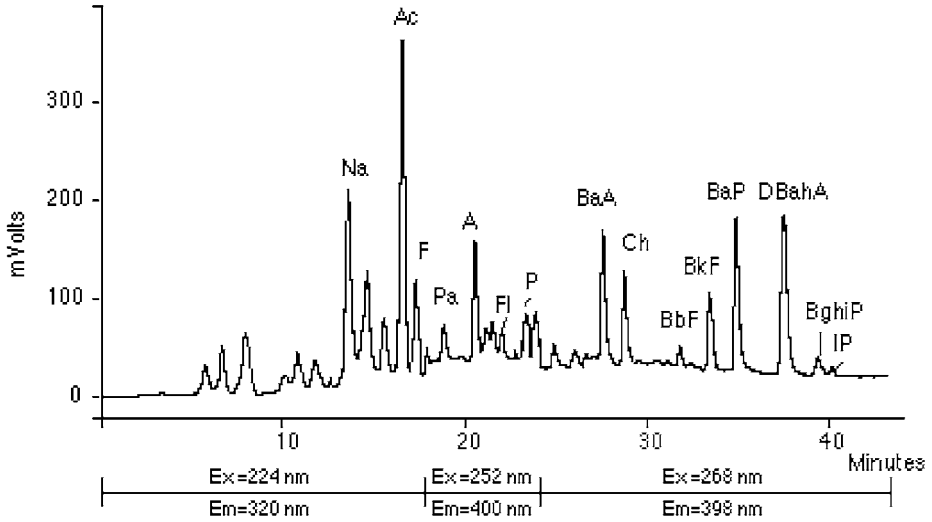

Figure 2. Spectrofluorometric chromatogram of cheese spiked with PAHs standard mixture in order to obtain the following concentrations (ppb): naphthalene (Na), 10.0; acenaphthene (Ac), 20.0; fluorene (F), 2.0; phenanthrene (Pa), 0.8; anthracene (A), 0.4; fluoranthene (Fl), 1.0; pyrene (P), 2.0; BaA, 1.0; Ch, 1.0; BbF, 0.4; BkF, 0.4; BaP, 1.0; DBahA, 4.0; benzo(ghi)perylene (BghiP), 1.6; and IP, 1.0. Below the chromatogram, the excitation (Ex) and emission (Em) detector conditions are indicated.

Table 1. Method Validation: Linearity, Quantification Limits, and Average of Recoveries at Three Concentrations Levels for the Different PAH in Cheese ($n = 3$ Determinations)

HMM PAH	amount added (μg/kg)	recovery (%)	linearity	LOQ (μg/L of solution)	LOQ (μg/kg of cheese)
BaA	0.06–1.0	76.5	0.999	1.50	0.03
Ch	0.06–1.0	95.8	0.996	1.50	0.03
BbF	0.04–0.4	93.7	0.951	2.00	0.04
BkF	0.02–0.4	84.6	0.999	0.60	0.01
BaP	0.06–1.0	83.5	0.998	1.50	0.03
DBahA	0.24–4.0	75.4	0.981	6.00	0.12
IP	1.0	85.2	0.995	45.00	0.90

ferences are present in cheese treated with different smoking generation material, in accordance with the fact that PAHs are not source specific (*4*).

Some quantitative differences are evident between the different wood used to produce smoke: except for fir and douglas wood, the total HMM PAHs content determined was lower than 2 μg/kg.

Among the wood materials, fir and douglas wood showed the highest levels of all of the single HMM PAHs considered, with the exception of BbF. The highest concentration of BbF was found in cheese smoked with pine wood even if this sample did not show a particular high total PAHs contamination. The level of BbF (0.44 μg/kg) is in accordance with the relatively high concentration of this compound among HMM PAHs found in the smoke of pine wood (*4*). Similar levels of BbF (0.41 μg/kg) were found also in cheese smoked with fir wood. The lowest concentration of HMM PAHs was found in cheese smoked with wheat straw, which is the real traditional procedure.

Nevertheless, it has to be considered that distributions and abundances of the smoke constituents are strongly dependent

Table 2. HMM PAH Content (μg/kg) of Diavoletto Cheese ($n = 3$ Determinations) Treated via Different Smoking Generation Materials in the Semiindustrial Cheese Factory (A)

cheese factory (A)	BaA	Ch	BbF	BkF	BaP	total
mix of wood shavings	0.40 (± 0.08)	0.38 (± 0.07)	traces	0.07 (± 0.02)	0.10 (± 0.01)	0.95
poplar wood shavings	0.22 (± 0.05)	0.16 (± 0.04)		0.04 (± 0.01)	0.08 (± 0.02)	0.50
straw and hay	0.13 (± 0.07)	0.15 (± 0.02)	traces	0.01 (± 0.02)	0.03 (± 0.01)	0.32
wheat straw	0.20 (± 0.04)	0.20 (± 0.05)		0.05 (± 0.02)	0.11 (± 0.02)	0.56
oak wood shavings	0.43 (± 0.09)	0.47 (± 0.10)	0.05 (± 0.01)	0.06 (± 0.01)	0.04 (± 0.01)	1.05
pine wood shavings	0.24 (± 0.05)	0.37 (± 0.01)	0.44 (± 0.17)	0.05 (± 0.04)	0.07 (± 0.01)	1.17
mahogany wood shavings	0.30 (± 0.08)	0.24 (± 0.05)	traces	0.07 (± 0.00)	0.13 (± 0.04)	0.74
fir wood shavings	2.71 (± 0.54)	1.75 (± 0.44)	0.41 (± 0.10)	0.33 (± 0.07)	1.01 (± 0.19)	6.21
douglas fir wood shavings	0.83 (± 0.25)	0.79 (± 0.19)	0.15 (± 0.02)	0.16 (± 0.02)	0.39 (± 0.09)	2.32
beech wood shavings	0.54 (± 0.13)	0.57 (± 0.15)		0.10 (± 0.02)	0.12 (± 0.02)	1.33
cardboard	0.69 (± 0.21)	0.50 (± 0.11)		0.11 (± 0.05)	0.16 (± 0.04)	1.46

Table 3. HMM PAH Content (μg/kg) of Diavoletto Cheese ($n = 3$ Determinations) Treated via Different Smoking Generation Materials in the Traditional Cheese Factory (B)

cheese factory (B)	BaA	Ch	BbF	BkF	BaP	total
mix of wood shavings	0.75 (± 0.27)	0.48 (± 0.10)		0.15 (± 0.02)	0.28 (± 0.03)	1.66
poplar wood shavings	0.82 (± 0.21)	0.54 (± 0.12)		0.07 (± 0.02)	0.16 (± 0.02)	1.59
straw and hay	0.61 (± 0.13)	0.42 (± 0.03)	traces	0.11 (± 0.07)	0.24 (± 0.03)	1.38
wheat straw	0.03 (± 0.01)	0.03 (± 0.01)		0.02 (± 0.00)	0.04 (± 0.01)	0.12
cardboard	0.83 (± 0.23)	0.68 (± 0.09)		0.18 (± 0.01)	0.31 (± 0.08)	2.00

Table 4. HMM PAH Content (μg/kg) in Liquid Smoke Flavoring and in Diavoletto Cheese after Smoke Flavoring and before Treatment

	BaA	Ch	BbF	BkF	BaP	total
cheese factory (A)						
liquid SFA	0.14 (± 0.10)	0.09 (± 0.02)		0.01 (± 0.01)	0.05 (± 0.05)	0.29
smoke-flavored cheese	0.10 (± 0.00)	0.18 (± 0.04)	traces	0.03 (± 0.00)	0.05 (± 0.01)	0.36
nonsmoked cheese	0.26 (± 0.03)	0.11 (± 0.05)		traces	traces	0.37
cheese factory (B)						
liquid SFA	0.77 (± 0.24)	0.69 (± 0.08)	traces	0.12 (± 0.00)	0.43 (± 0.03)	2.01
smoke-flavored cheese	0.44 (± 0.29)	0.18 (± 0.06)		0.02 (± 0.03)	0.08 (± 0.01)	0.72
nonsmoked cheese	0.22 (± 0.00)	0.13 (± 0.02)		0.03 (± 0.03)	0.03 (± 0.02)	0.41

on combustion conditions (*1*, *5*, *13*). In fact, comparing the results obtained with the same kind of wood, the cheeses smoked in the semiindustrial factory showed a lower HMM PAHs contamination. This is probably due to the more controlled combustion conditions.

The results obtained in cheese smoked by experimental purpose with cardboard did not show a particularly high contamination, but the potential health risk can derive from other components produced by the combustion of synthetic materials such as glue, ink, and other residues of treatments.

Smoke-Flavored Cheese. Table 4 shows the HMM PAHs content determined in liquid smoke flavoring, in smoke-flavored cheese, and in cheese before treatment. The two SFAs analyzed showed differences in the HMM PAHs content. The values obtained were in agreement with those declared by the two producers in the label.

As far as the data obtained with aromatized cheeses are concerned, the HMM PAHs content reflects the different contamination of the two liquid SFAs although to a lower extent (*13*). In both cases, the BaP level was higher than 0.03 μg/kg. However, it has to be considered that PAHs are environmental contaminants; therefore, they are present also in nonsmoked food such as leaves of vegetables, grains, fruits, and vegetable oils (*3*, *16*, *17*).

As shown in **Table 4**, it was possible to determine the limited presence of HMM PAHs also in cheese before treatment. These are very stable compounds; therefore, they can migrate through the food chain into hydrophobic compartments. PAHs have been found in grass and milk sampled near potential contaminating sources (*18*, *19*).

CONCLUSIONS

This study showed that the smoking process of Diavoletto cheese implies a limited contamination with HMM PAHs. Moreover, if we exclude conifer woods, the total HMM PAHs contamination in cheese was lower than 2 μg/kg, which is much lower than the 5 μg/kg limit proposed by the German Society of Fat Science. In particular, the BaP content was always below 1 ppb, which is the limit proposed by the EU. A "zero tolerance" level for ubiquitous environmental contaminants such PAHs is inconceivable; in fact, HMM PAHs were already detected before processing.

It would be advisable to issue guidelines for the smoking process, like it has been done in the EU for the SFAs. No PAH limits are set for smoked food. Limits for the other "probable human carcinogen" PAHs should be enforced, beside the one for BaP for smoke-flavored food set by the EU. Only the complete evaluation of the "probable human carcinogen" PAHs will properly secure the safety of smoked or smoke-flavored foods.

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