JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Levels of Benzo[*a*]pyrene (BaP) in "Mozzarella di Bufala Campana" Cheese Smoked According to Different Procedures

ANIELLO ANASTASIO,* RAFFAELINA MERCOGLIANO, LUCIA VOLLANO, TIZIANA PEPE, AND MARIA LUISA CORTESI

Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti, Sezione Ispezione, Facoltà di Medicina Veterinaria, Università degli Studi di Napolii Federico II, Via Delpino 1, Napoli 80137, Italy

The content of benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon, was determined by HPLC-FL in "mozzarella di bufala campana" cheese, a stretched cooked cheese, either experimentally smoked according to traditional procedures, using straw, cardboard, and wood shavings or aromatized with smoke flavoring. The BaP residues, researched also in cheese samples sold at retail, were detected in the rind, in the core, and in the slice (outer and inner parts). In the cheeses experimentally smoked with straw and cardboard the BaP levels, ranging from 0.38 to 2.12 μg kg^{-1} and from 0.46 to 2.40 μg kg^{-1}, respectively, were statistically higher than those of the cheeses smoked with wood shavings and aromatized with liquid smoke (from 0.19 to 0.80 μg kg^{-1} and from 0.18 to 0.50 μg kg^{-1}, respectively). However the cheeses treated with liquid smoke flavor showed a BaP content exceeding the level allowed by the European Union. In the samples sold at retail, smoked with straw, values were lower than those obtained from samples smoked experimentally with the same combustible. This is probably due to different smoking technologies among the several provinces of the Protected Designation of Origin (PDO) area. PDO is a term used to characterize foodstuffs produced and prepared in a given geographical region by the means of a recognized process. A standardization of the traditional smoking procedures and an improvement of liquid smoke purification treatments are recommended for mozzarella cheese.

KEYWORDS: Smoked mozzarella cheese; benzo[*a*]pyrene; smoking procedures; HPLC-FL

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds characterized by a benzoic nucleus and resulting from the incomplete combustion or pyrolysis of organic materials. Some of these hydrocarbons are known to be carcinogenic (*1*−*3*). Benzo[*a*]pyrene (BaP) is the most well-known and studied member of these compounds because it is one of the most potent PAH animal carcinogens (*1*, *2*). Data on the carcinogenity of PAHs are mostly focused on respiratory carcinogenesis. However, neoplasms in the stomach and mammary gland induced in experimental animals following administration of PAHs by an oral route are reported in the literature (*4*, *5*). The Total Human Exposure to Environmental Substances (THEES) study had shown that people in the general population without substantial environmental and occupational exposure had a higher exposure to BaP by food ingestion than by inhalation (*6*). Some technological processes, such as boiling and smoking, can increase the PAH levels (*7*−*9*). In the literature are reported a lot of data on PAH contamination due to the smoking processes of different food products (*8*−*11*). "Mozzarella di bufala campana" has been included among the Protected Designation of Origin (PDO) cheeses since 1996 (*12*). PDO is a term used to characterize foodstuffs produced and prepared in a given geographical region by the means of a recognized processes. Mozzarella cheese may be smoked to obtain an additional flavor provided that the smoking procedure is performed "according to a natural and traditional technology" (*13*). A traditional smoking is generally carried out using a metal container provided with a cover, which is placed on the upper edge of the container, to smooth the flame. The resulting smoke reaches the cheese placed on or suspended at gratings of the upper edge of the smoking drum. Sometimes straw and wood shavings are used as combustibles. However, the possibility of an unapproved use of cardboard should be considered.

Currently in Italy no limit values exist regarding BaP and other PAHs in smoked foods. As BaP has been frequently considered a marker for PAH contamination (*5*, *9*, *11*), the aim of this paper was to study the distribution of this hydrocarbon in mozzarella di bufala campana cheese smoked according to different technologies and to evaluate the levels of BaP residues in samples of mozzarella cheese sold at retail in southern Italy.

* Author to whom correspondence should be addressed (telephone +39 081 459560; fax +39 081 458683; e-mail anastasi@unina.it).

10.1021/jf049566n CCC: $27.50
Published on Web 06/16/2004

Table 1. Smoking Chamber Parameters Settled to Smoke with Wood Shavings

period	temp (°C)	time (min)	air speed (m/s)
1	50	3	2
2	46–48	20	4
3	44–46	7	4

MATERIALS AND METHODS

Experiment 1. Thirty-two samples of mozzarella di bufala campana cheese, a stretched cooked cheese [200−250 g, 52 g of fat and 35 g of dry matter per 100 g (minimum content)], previously analyzed and with BaP values under the detection limit, produced in the same dairy establishment, were placed in a 10% saline solution for a few minutes and divided randomly into four groups, each consisting of eight samples. The samples of the first three groups were then smoked with straw, cardboard, and wood shavings, respectively. The mozzarella cheeses of the fourth group were aromatized with liquid smoke flavor.

The smoking procedures were similar for straw and cardboard, which were both placed at the bottom of a metal tank (20 L). The cheeses were hung by means of a rope on the top of the tank and then exposed for a maximum of 10 min to the smoke obtained by choking the fire. When wood shavings were used as combustible, smoke was produced in a smoking chamber where the parameters were settled as described in **Table 1**.

All smoked mozzarella cheeses were washed in water and then placed in brine.

Aromatization with liquid smoke flavoring was performed by dipping the samples for 10 min in stainless steel tanks containing a 50% liquid smoke flavor solution. The cheeses were then dried for 2 h at 20 °C.

All samples were transported under refrigeration and stored at −20 °C until analyzed. Each cheese was examined for BaP residues in the rind, in the core, and in a slice comprising the inner and outer parts of the cheese cut in the central area of the sample. Each experiment was repeated three times. On the whole 288 samples were analyzed in duplicate.

Experiment 2. Forty-eight samples of smoked mozzarella di bufala campana cheese, sold in 19 retail shops annexed to the semi-industrial dairy establishments and located in PDO area of southern Italy, were analyzed. Ten establishments were sampled three times, nine only twice. The cheeses were all smoked, using straw as combustible, according to each manufacturer's own procedures, which include also the use of smoke generators with smoke purification systems. After smoking, the samples were washed briefly, placed in brine, and transported under refrigeration to the laboratory, where they were stored at −20 °C until analyzed. BaP residues were researched in the rind, in the core, and in a slice comprising the outer and the inner parts of the cheese, also in this experiment. On the whole 144 samples were analyzed in duplicate.

Chemicals and Reagents. Solvents and reagents (HPLC grade) were from Carlo Erba (Milano, Italy). All of the analytical grade solvents were submitted to further distillation to reduce the PAH external contamination. Water was obtained from a Milli-Q plus ultrapure water system (Millipore, Bedford, MA). Solid-phase extraction (SPE) was performed with Isolute (silica 500 mg/3.0 mL) cartridges obtained from Supelco (Bellefonte, PA).

BaP standard (CAS Registry No. 50328) was obtained from Sigma (St. Louis, MO).

Apparatus. An automated SPE system, Aspec XL Gilson (Worthington, OH), was used for the SPE cleanup. The HPLC analyses were performed with a JASCO (Tokyo, Japan) instrument equipped with a ternary pump (Pu-980), an autosampler (AS-2055 PLUS), and a fluorescence detector (FP 210). The HPLC column was an Enviroseep-PP 125 × 4.6 mm i.d., with a packing of 4.6-μm particles (Phenomenex).

Sample Preparation. Mozzarella cheese samples were extracted according to the method of Bosset et al. (*1*) modified as follows: 1 M KOH etanolic solution (10 mL) was added to 2 g of sample previously homogenized in a 50-mL glass centrifuge tube. The mixture was placed for 3 h in a water bath (80 °C) and then cooled at room temperature; 10 mL of H_2O and 20 mL of cyclohexane were added, and the mixture was vortexed for 5 min and then centrifuged for 15 min at 4000*g*. The supernatant layer was poured from the tube, through a folded filter paper, into a 100-mL flat-bottom flask. The sample was re-extracted as previously described with 20 mL of cyclohexane. The combined extracts were dried over anhydrous Na_2SO_4, reduced to 1 mL by rotavapor (45 °C), and evaporated to dryness under nitrogen. The residue dissolved in acetonitrile (2 mL) was applied to a SPE cartridge pretreated with acetonitrile (5 mL). BaP was eluted with two portions of acetonitrile (3 mL), and the eluate was evaporated to dryness under nitrogen. The residue was dissolved in 500 μL of acetonitrile.

Chromatographic Method. Twenty microliters of acetonitrile solution were injected into the chromatographic system.

BaP was analyzed in isocratic elution at 1.0 mL/min at room temperature, with a mobile phase consisting of acetonitrile/water (88:12, v/v). Excitation and emission wavelengths were 294 and 404 nm, respectively.

Method Calibration. To check the linearity were analyzed standard mixtures at five concentration levels (0.05, 0.1, 0.5, 1, and 10 μg/L). The above-mentioned mixtures were obtained from a working solution of 50 μg/L. A calibration curve was constructed by plotting mean ($n = 3$) peak area (y) versus standard concentrations in micrograms per liter (0.05−10) (x).

To determine accuracy, 2-μl portions of 50, 500, and 1000 μg L^{-1} standard solutions were added to negative control (mozzarella samples not smoked) to give fortification levels of 0.05, 0.5, and 1 μg kg^{-1}, respectively. Fortified samples were extracted and determined as described earlier.

The precision of the method, expressed as within-day repeatability, was determined by analyzing samples ($n = 3$ determinations) spiked with BaP standard solutions (0.05−1.0 mg kg^{-1}). The detection limit (LOD) and the limit of quantification (LOQ) were calculated following AOAC guidelines (*14*). The conversion of LOD and LOQ to micrograms of BaP per kilogram of cheese results from the injection volume and the mass of sample analyzed. Recovery was measured using fortified samples ($n = 6$ replicates) each at two concentrations (0.05 and 0.5 μg kg^{-1}).

The specificity was confirmed by analysis of blank samples. No interferent peak eluted at the same retrention time of BaP.

Data were submitted to analysis of variance (ANOVA) of the SAS procedure using the model $y_{ij} = \mu + ST_i + \epsilon_{ij}$, where μ = mean, ST_i = smoke treatments (i, 1−4), and ϵ_{ij} = residual error (*15*). Values were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

The analytical method used to extract and quantify the cheese BaP concentrations was shown to be adequate. In fact, the regression analysis of the data showed that BaP responses were linear over the range of the examined concentrations. The correlation coefficient obtained exceeded 0.99, demonstrating a good linearity. BaP recoveries were 84.4 and 88.9% for the two amounts added (see Method Calibration). The LOD and the LOQ were 0.0058 and 0.021 μg kg^{-1}, respectively.

In the first experiment BaP residues were detected in all samples, and values (mean of the three trials) varied according to the smoking procedure and sample typology, from 0.50 to 2.40 μg kg^{-1} in the rind, from 0.22 to 1.03 μg kg^{-1} in the slice, and from 0.18 to 0.46 μg kg^{-1}in the core (**Table 2**; **Figure 1**). The highest BaP value detected was 2.40 μg kg^{-1}, found in the rind of a mozzarella smoked with cardboard.

In the second experiment BaP residues were found in $n =$ 48 rind (100%) and in $n =$28 slice samples (58%), at levels ranging from 0.05 to 0.75 μg kg^{-1} and from 0.11 to 0.33 μg kg^{-1}, respectively. Only in two core samples (4%) were detected BaP residues at levels of 0.10 μg kg^{-1}. In the rind of these samples the hydrocarbon levels were 0.55 and 0.64 μg kg^{-1}. The percentage of positive samples is the same, and the levels of contamination were very similar among the three sampling periods (**Table 3**).

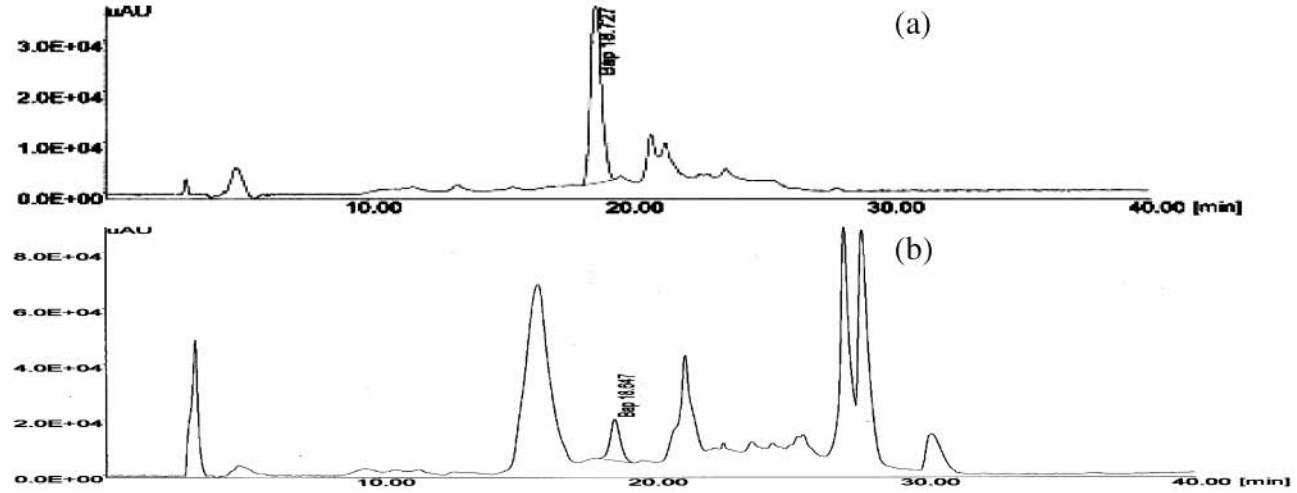

Figure 1. Chromatograms of (**a**) 50 ng mL^{-1} benzo[*a*]pyrene standard and (**b**) mozzarella cheese experimentally smoked with straw.

Table 2. BaP Levels Detected in the Core, Slice, and Rind of Mozzarella di Bufala Campana Cheese Smoked According to Different Procedures[a] ($n = 96$)

	BaP, mean ± SD (μg kg^{-1})		
smoking procedure	rind	slice	core
straw	2.12Ab ± 1.40	1.03A ± 0.75	0.38Ab ± 0.19
cardboard	2.40Aa ± 1.23	1.01A ± 0.48	0.46Aa ± 0.20
wood shavings	0.80Bc ± 0.35	0.30B ± 0.15	0.19B ± 0.07
smoke flavor	0.50Bd ± 0.33	0.22B ± 0.20	0.18B ± 0.12

[a] In each column, different letters indicate significant difference (A, B, $P < 0.01$; a–d, $P < 0.05$) among different smoke treatments

Table 3. BaP Levels Found in Smoked Mozzarella di Bufala Campana Cheese Taken from Retail Analyzed during Three Sampling Periods (See Text)

sample	positive samples (%)	min–max (μg/kg^{-1})	mean	SD
first sampling ($n = 19$)				
rind	19 (100)	0.14–0.62	0.34	0.15
slice	11 (58)	0.11–0.24	0.14	0.03
core	1 (5)	0.10		
second sampling ($n = 19$)				
rind	19 (100)	0.05–0.64	0.36	0.17
slice	11 (58)	0.12–0.33	0.22	0.07
core	1 (5)	0.15		
third sampling ($n = 10$)				
rind	10 (100)	0.16–0.75	0.46	0.21
slice	6 (60)	0.14–0.31	0.20	0.07
core				

The cheese BaP contents are the consequence of the variables involved in the smoking process, including the kind of combustible, the combustion temperature, and the length of smoking.

The levels detected in the cheeses experimentally smoked with straw or cardboard, following traditional procedures, were statistically higher than those found in the products smoked following the other two procedures (**Table 2**). This may be due to the fact that smoke purification was not performed and the combustion conditions are not as controlled as the smoking chambers are. In addition, the inside walls of the tanks, which were often fouled by tar residues originating from the previous processing, were not cleaned. The BaP concentrations found in the cheeses aromatized with smoke flavoring, although lower than those found in samples smoked with the remaining procedures, demonstrate that the smoking flavor agents (SFAs) may contain hydrocarbon residues. We did not analyze the smoke flavorings, but the mozzarella samples used had no BaP levels detectable before treatment. In a previous study, Yabiku et al. (*16*) found BaP levels higher than the maximum level recommended by FAO/WHO (10 μg kg^{-1}) in 3 of 11 samples of SFAs. The use of these aromas is allowed by EU Directive, provided that the BaP and benzoanthracene (BaA) residues do not exceed the level of 0.03 μg kg^{-1} in the finished products (*17*). The BaP levels detected in mozzarella aromatized with smoke flavoring were higher than the above-mentioned level. Several years ago the proper procedure for the production of SFAs, including purification steps, was correlated to their different uses. SFAs to be used only for surface treatments, for example, did not require the removal of substances responsible for the desired product color (*18*). In our opinion the efficient removal of hazardous compounds is needed also when SFAs are used for surface treatment because sometimes the surface of the product may be ingested. Recently, the European Council by Regulation CE 2065/2003, concerning the SFAs used or intended for use in or on foods, fixed the maximum levels in the smoking aromas of BaP and (BaA) at 10 and 20 μg kg^{-1} and established a procedure for their safety assessment. The producers shall have to provide detailed information on the materials used and the production methods and have to guarantee the tracking of SFAs. Authorization will be granted by the European Food Safety Authority for a period of 10 years and shall be amended according to the latest scientific and technical knowledge (*19, 20*).

The BaP concentrations found during the second experiment fall within the same range found by Bosset et al. (*1*) in $n = 33$ samples of cheese ($n = 24$ of Etivaz and $n = 9$ of different kinds of Gruyère) and are lower than the maximum accepted level of 1 μg kg^{-1} fixed by some European countries for some kinds of foods, smoked cheeses included, a level that the EU is planning to set as a limit in smoked foods (*1*, *9*, *10*). No differences were found in the levels of contamination among the three sampling periods (**Table 2**). The values found in the cheeses smoked with straw and sampled in retail shops annexed to the dairy establishments were lower than those obtained from samples smoked experimentally, using traditional procedures, with the same combustible. This is probably related to the use of a nonstandardized smoking technology, mainly based on the manufacturer's personal evaluation, which might include smoke purification steps. It is interesting that differences in smoking

procedures exist among manufacturers also within the single provinces of the PDO area.

The concentrations detected in the present study suggest that the smoking practices currently used in semi-industrial dairy establishments are effective in controlling the hydrocarbon deposition. However, the presence of BaP residues in the rind samples deserves attention as the smoked mozzarella rind is often eaten by Italian consumers. Therefore, the identified levels should be considered when the consumer risk is evaluated, which is known to be related also to the amount of contaminated food and the frequency of ingestion (*7*, *8*). Although cheeses are thought to give a limited contribution to the total BaP human intake (*21*), the BaP levels found in mozzarella cheeses experimentally treated, in particular those detected in cheeses smoked with cardboard, deserve careful attention. Moreover, the use of cardboard is a cause for additional concern, owing to the paint and glue residues that may release undesirable substances during smoking. The use of cardboard as a combustible is not allowed, but its illegal use in dairy establishments cannot be excluded. At the present time no PAH and BaP levels have been set by the EU for smoked foods, but the problem is under discussion. In agreement with Pagliuca et al. (*10*) the issue of EU guidelines for the smoking process, as well as for liquid smoke production, would be advisable. A standardization of the traditional smoking procedures and an improvement of liquid smoke purification treatments are recommended for mozzarella cheese smoked according to traditional procedures.

LITERATURE CITED

(1) IARC. *Evaluation of the Carcinogenic Risk of Chemicals to Humans, Polynuclear Aromatic Compounds*: *Chemical, Environmental and Experimental Data*; Monograph 32; IARC: Lyon, France, 1983; p 1.

(2) Integrated Risk Information System (IRIS). *Summaries for Benzo[a]anthracene, Chrysene, Benzo[b]flouranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Dibenzo(a,h)anthracene and Indeno(1,2,3-cd)pyrene*; U.S. Environmental Protection Agency: Washington, DC, 2002; http:/www.epa.gov/iriswebp/iris/index.html.

(3) Howard, J. W.; Fazio, T. Rewiew of polycyclic aromatic hydrocarbons in foods. *J. Assoc. Off. Anal. Chem*, **1980**, *63*, 1077−1104.

(4) IARC. *Evaluation of the Carcinogenic Risk of Chemicals to Humans, Polynuclear Aromatic Compounds: Overall Evaluation of Carcinogenicity: an Updating*; Monograph 1-42; IARC: Lyon, France, 1987; p 7.

(5) El Bayoumy, K.; Chae, Y. H.; Upadhyaya, P.; Rivenson, A.; Kurtzke, C.; Reddy, B.; Hecht, S. S. Comparative tumorigenicity of benzo[*a*]pyrene, 1-nitropyrene and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine administered by gavage to female CD rats. *Carcinogenesis* **1995**, *16*, 431−434.

(6) Butler, J. P.; Post, G. B.; Lioy, P. J.; Waldman, J. M.; Greenberg, A. Assessment of carcinogenic risk from personal exposure to benzo[*a*]pyrene in the total human environmental exposure study (THEES). *J. Air Waste Manag. Assoc*. **1993**, *43*, 970−977.

(7) Bories, G. In *Toxicologie et Sécurité des Alimentes*; Technique et Documentation, Lavoisier: Paris, France, 1983; pp 362−369.

(8) Bosset, J. O.; Bütikofer, U.; Dafflon, O.; Koch, H.; Scheurer-Simonet, L.; Sieber, R. Teneur en hydrocarbures aromatiques polycycliques de fromages avec et sans flaveur de fumée. *Sci. Aliments* **1998**, *18*, 347−359.

(9) Garcia Falcon, M. S.; Gonzales Amigo, S.; Lage Yusty, M. A.; Simal Lozano, J. Determination of benzo[*a*]pyrene in some Spanish commercial smoked products by HPLC-FL. *Food Addit. Contam.* **1999**, *16*, 9−14.

(10) Pagliuca, G.; Gazzotti, T.; Zironi, E.; Serrazanetti, G. P.; Mollica, D.; Rosmini, R.; Determination of high molecular mass polycyclic aromatic hydrocarbons in a typical Italian smoked cheese by HPLC-FL. *J. Agric. Food Chem.* **2003**, *51*, 5111−5115.

(11) Storelli, M. M.; Giacominelli Stuffler, R.; Marcotrigiano, G. O. Polycyclic aromatic hydrocarbons, polychlorinated biphenyls chlorinated pesticides (DDTs), hexachlorocyclohexane and hexachlorobenzene residues in smoked seafood, *J. Food Prot.* **2003**, *66*, 1095−1099.

(12) EEC Regulation 1107/96. *Off. J. Eur. Communities* **1996**, *No. 148* (June 21).

(13) DPCM 10 May 1993. *Off. J. Eur. Communities* **1993**, *No. 219* (Sept 17).

(14) AOAC Peer−Verified Methods Program. *Manual on Policies and Procedures*; AOAC International: Gaithersburg, MD, 1994.

(15) Statistical Analysis System Institute, Inc. *SAS/STAT User's Guide*, 6.03 ed.; SAS Institute: Cary, NC, 1989.

(16) Yabicu, H. Y.; Martins, M. S.; Takahashi, M. Y. Levels of benzo(α)pyrene and other polycyclic aromatic hydrocarbons in liquid smoke flavour and some smoked foods. *Food Addit. Contam*. **1993**, *10*, 399−405.

(17) Council Directives 88/388/ CEE and 91/71/CEE. "The approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production. *Off. J. Eur. Communities* **1988**, *No. L184* (July 15); **1991**, *No. L143* (May 25).

(18) Gorbatov, V. Liquid smokes for use in cured meats. *Food Technol.* **1971**, *25*, 71−77.

(19) IP/02/1094, Food: Commission proposes EU-wide safety assessment and authorisation of smoke flavourings. Brussels, Belgium, July 18, 2002; http://europa.eu.int/comm/food/fs/sfp/additflavor/flavourings/index_en.html.

(20) 2065/2003/CE: European Parliament and Council Regulation of Nov 10, 2003, on the smoke flavourings used or intended for use in or on foods. *Off. J. Eur. Communities* **2003**, *No. L309/1* (Nov 26).

(21) Kazeroumi, N.; Sinha, R.; Hsu, C. H.; Geenberg, A.; Rothman, N. Analysis of 200 food items for benzo[*a*]pyrene and estimation of its intake in an epidemiological study. *Food Chem. Toxicol*. **2001**, *39*, 423−436.

Received for review March 16, 2004. Revised manuscript received May 6, 2004. Accepted May 8, 2004.

JF049566N