Optimization and Validation of an Extraction Method for the Analysis of Polycyclic Aromatic Hydrocarbons in Chocolate Candies

Rupender Kumari, Prashant Chaturvedi, Nasreen G. Ansari, Ramesh C. Murthy, and Devendra K. Patel

Abstract: Chocolate is a key ingredient in many foods such as milk shakes, candies, bars, cookies, and cereals. Chocolate candies are often consumed by mankind of all age groups. The presence of polycyclic aromatic hydrocarbons (PAHs) in chocolate candies may result in health risk to people. A rapid, precise, and economic extraction method was optimized and validated for the simultaneous determination of polycyclic aromatic hydrocarbons in chocolate candy by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) as a confirmatory technique. The method was optimized by using different solvents for liquid–liquid extraction, varying volume of demulsifying agent, and quantity of silica gel used for purification. The HPLC separation of 16 PAHs was carried out by C-18 column with mobile phase composed of acetonitrile : water (70 : 30) in isocratic mode with runtime of 20 min. Limit of detection, limit of quantification (LOQ), and correlation coefficients were found in the range of 0.3 to 4 ng g⁻¹, 0.9 to 12 ng g⁻¹, and 0.9109 to 0.9952, respectively. The exploration of 25 local chocolate candy samples for the presence of PAHs showed the mean content of benzo[a]pyrene as 1.62 ng g⁻¹, which representing the need to evaluate effective measures to prevent more severe PAHs contamination in chocolate candies in future.

Keywords: activated silica gel column clean-up, chocolate, GC-MS, liquid–liquid extraction (LLE), polycyclic aromatic hydrocarbon, RP-HPLC

Practical Application: Chocolate is one of the most favorite food items among people, especially children. Chocolate candies are often consumed by mankind of all age groups. Chocolate candies are often consumed by children in large quantities. The presence PAHs in chocolate candies may result in health risk to people. In the present study, a precise and cost effective rapid method was employed for the determination of PAHs, which can be employed for daily routine analysis of PAHs in chocolate products.

Introduction

Chocolate is a key ingredient in many food items, such as milk shakes, candies, bars, cookies, and cereals and is also considered as one of the most favorite food items among people, especially children. The common ingredients of chocolates are cocoa solid, cocoa butter, sugar, glucose, wheat flour, buffering agents, carbohydrates (edible starches), proteins, alkaloids, minerals, fat, and aroma or flavoring substances (Belitz and others 2004). The world cocoa consumption progressed at a rate of 2.7 percent per annum and reached to 2.83 million tons during the previous decade, that is, 1988 to 1990 to 2000 (Medium-term prospects for agricultural commodities 2010). Chocolate requires complex procedures to produce, which involves harvesting of cocoa, fermenting cocoa to cocoa beans and shipping the cocoa beans to the manufacturing factory for cleaning, drying, roasting, winnowing, grinding, molding, blending, and conching which may produce polycyclic aromatic hydrocarbons (PAHs) (The World Atlas of Chocolate 2003). The term PAHs commonly refers to a large class of organic compounds containing 2 or more fused aromatic rings made up of carbon and hydrogen atoms. A high amount of PAHs are emitted from incomplete combustion or high temperature pyrolytic processes involving fossil fuels, forest fires, volcanoes or hydrothermal processes, processing coal and from motor vehicle exhaust (Poster and others 2006; Wenzl and others 2006; Simon and others 2008). PAHs have high melting and boiling-points, low vapor pressure, and very low water solubility, which tend to decrease with increasing molecular mass. More than 100 PAHs have been characterized in nature, 16 of which were classified as “priority pollutant” according to U.S. Environmental protection agency (EPA) (Compendium method TO-13A 1999). The Intl. Agency for Research on Cancer (IARC) concluded that a number of PAHs are genotoxic carcinogens in nature, out of which, benzo[a]pyrene (BaP) and dibenzo[a, h]anthracene (DbahA) were reported as the most carcinogenic PAHs (IARC 1983, 1987). FAO/WHO expert committee on food additives declared that amount of BaP in food should not be exceeding than 10 ppb (JECFA 2005).

Food can be contaminated by PAHs that are present in air, soil, or water or are formed during processing (during drying or...
smoking) and cooking (during grilling, roasting, or frying), which permit the direct contact between food and combustion products; these are important sources of PAH contamination for seeds, edible oils, and meat and dairy products (EFSA 2008). Due to lipophilic nature of PAHs, fats, and oils can be especially susceptible to PAHs contamination. After a survey on PAHs in food products, European Food Safety Authority (EFSA 2008) has reported that out of all analyzed food products, benzo(a)pyrene was consistently found in barbequed meat, dried tea, chocolate, cocoa butter, and hivale mollusks. In command to protect public health, the EU legislation has fixed maximum levels of 5 μg kg⁻¹ benzo(a)pyrene in cocoa butter intended for direct human consumption or use as an ingredient in foods (European Commission 2006).

The level of PAHs in various food items depends upon the heat source (coal, wood, gas, and so on), temperature, particulate material generated during combustion, and upon the type of processing method, that is, steaming, roasting, smoking, grilling, and so on, used for food preparation. The occurrence and level of PAHs in different type of food products, that is, in plant food (Zohair and others 2006; Rey-Salgueiro and others 2008a), milk (Kishikawa and others 2003), infant’s food, toasted bread, and sausage (Nieva-Cano and others 2001; Garcia-Falcon and Simal-Gandara 2005; Rey-Salgueiro and others 2008b; Rey-Salgueiro and others 2009a), coffee and tea (Kayali-Sayadi and others 1998; Garcia-Falco and others 2005; Houessou and others 2005), smoked meat (Chiu and others 1997; Djinovic and others 2008), and seafood (Jänská and others 2004; Perugini and others 2007; Rey-Salgueiro and others 2009b) has been demonstrated by many researchers. However, limited data are available on the PAHs content in different types of chocolate and chocolate products. Dennis and Lodovici (1991, 1995) had analyzed different types of chocolate samples and found BaP content in the range of 0.13 to 0.32 μg kg⁻¹ and 0.33 μg kg⁻¹, respectively. In the year 2001, BaP content of 0.18 μg kg⁻¹ was reported in a chocolate candy by Kazerouni and others (2001). A survey of SCF-PAHs in food products on Irish market by the Food Safety Authority of Ireland, the BaP contents were found in the range of 0.06 to 0.30 μg kg⁻¹ in 16 analyzed chocolate samples (Food Safety Authority of Ireland 2006). In the year 2007, a high concentration of BaP of 10 μg kg⁻¹ was reported in a sample of bitter chocolate by Warendt (2007). Total of 40 samples of different type of chocolates were analyzed by Ziegenhals and others (2009) and found the BaP contents in the range of 0.7 to 0.63 μg kg⁻¹.

As per the scientific literature available till the date, analysis of 16 priority PAHs has not been reported in chocolate candies. Thus, it was essential to develop an extraction method to explore the occurrence of the PAHs in chocolate candies. The main objective of our study was to establish an easy, sensitive, rapid, accurate, economic, and efficient extraction method for simultaneous determination of 16 PAHs in chocolate candy samples with their analysis by RP-HPLC and confirmation by GC-MS.

Materials and Method

Sample collection

Commercially available chocolate candy samples were purchased from the local market of Lucknow city, India. The candy samples were stored at 4 °C until processed for extraction of PAHs.

Chemicals and equipment

Standards of Naphthalene (Nap), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (FluA), Pyrene (Pyr), Benzo[a]anthracene (BaA), Chrysene (Chr), Benzo[k]fluoranthene (BkF), Benzo[k]fluoranthene (BkF), Benzo[a]pyrene (BaP), Indeno[1,2,3-c,d]pyrene (Ind), Dibenzo[a,h]anthracene (DBahA) and Benzo[g,h,i]perylene (BghiP) were procured from Sigma Aldrich (St. Louis, Mo., U.S.A.), all with purity higher than 98%.

All solvents used, that is, acetonitrile, methylene chloride (DCM), ethanol, n-hexane, n-pentane, and cyclohexane were obtained in HPLC grade from Merck Pvt. Ltd., Mumbai, India. Anhydrous sodium sulfate and silica gel were provided by SISCO research laboratories Pvt. Ltd., Mumbai, India. Chromatographic glass column (450 × 40 mm) was procured from Boroil India Ltd., Bharuch, Gujarat, India. Ultrapure water was manufactured in-house with a water purification system (Milli-Q synthesis Elix-10, Millipore Corp., Mass., U.S.A.). Nitrogen Evaporator (TurboVap®LV) was obtained from Caliper Life Sciences, Eln Street, Mass., U.S.A.

Validation parameters

Limit of detection (LOD) and limit of quantification (LOQ). The limit of detection of an individual analyte is the lowest amount of analyte in a sample that can be detected with acceptable certainty but not quantified as an exact value. The limit of quantification of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy. A signal-to-noise ratio (S/N) of 3 was for estimating LOD and signal-to-noise ratio of 10 was used for estimating LOQ. Peak-to-peak noise around the analyte retention time was measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio was estimated. The noise magnitude was measured by auto-integrator of the instrument.

Linearity. The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations. The linearity was evaluated by regression analysis. Calibration curves for individual PAH were constructed by plotting average peak area against concentration and regression equation was computed. To establish linearity, a minimum of 5 concentrations is recommended.

Recovery percentage. Recovery percentage was calculated by the formula as b-a/c × 100 where (b) is the amount of analyte found after the addition of standard solution, (a) is the amount of the analyte found before the addition of standard solution, and (c) is the amount of standard compound added.

Standard preparation. PAHs stock solutions were prepared by weighing exactly 10 mg of each standard, dissolved separately in 100 mL of acetonitrile. Mixed external reference standard solution was prepared by diluting stock solution of each compound at different concentrations in amber color volumetric flask (to avoid light exposure) and stored at 4 °C whenever not in use.

Sample preparation Three grams of each candy sample were weighed, diluted with 30 mL of de-ionized water and the resulting solution was homogenized by shaking for 12 h.

Liquid–liquid extraction. The diluted solution was extracted 3 times with 5 mL n-Hexane, followed by addition of 0.5 mL of ethanol for emulsification. The extract obtained were dried under nitrogen flow and re-dissolved in 2 mL of cyclohexane.
Sample purification. The sample's extract may contain substantial amounts of material other than PAH, which may interfere with the analytical determination. To avoid these interferences, purification has been carried out using column chromatography. The chromatographic column was prepared with 3 g of activated silica gel sandwiched in 0.5 g anhydrous sodium sulfate. The column was pre-eluted with 10 mL n-pentane and just before the exposure of sodium sulfate layer to air, 2 mL of cyclohexane extract was transferred onto the column. The sample vial was washed with additional 2 mL cyclohexane and transferred to the column. The aliphatic fraction was eluted with 10 mL n-pentane and discarded.

The aromatic fraction was eluted with 15 mL mixture of DCM : n-pentane (2 : 3, v/v). The whole eluent was collected, evaporated to dryness under a gentle gas stream of nitrogen, and the final volume was made up with 0.5 mL acetonitrile, filtered through 0.45 μm sized filter. An aliquot of 50 μL was injected and analyzed by HPLC system.

Method optimization. The HPLC method used for the determination of PAHs provided satisfactory separation of 16 PAHs within 20 min. For the optimization of extraction method, parameters including selection of solvent for extraction, quantity of de-emulsifying agent, and column composition were studied step by step and optimized.

All optimization steps were carried out by weighing 3 g of chocolate candy sample, diluted in 30 mL de-ionized water in a 100 mL conical flask. An appropriate volume of mixed PAHs standard solution at 3 levels concentration, that is, 4 to 44.8 ng g⁻¹, 8 to 89.6 ng g⁻¹, and 16 to 179.2 ng g⁻¹, were added to the chocolate candy samples and kept at 4 °C for at least 24 h for stabilization.

To select the optimum solvent for PAHs, extractions were performed by the solvents of various polarities. The spiked chocolate samples were extracted with three individual solvents (n-hexane, DCM, cyclohexane) and with 3 different combination of solvents (n-hexane : DCM [1 : 1, v/v], n-hexane : DCM [1 : 2, v/v], n-hexane : DCM [2 : 1, v/v]). Out of these solvents, n-hexane and n-hexane : DCM (1 : 1) were found to be better for extraction of both low molecular mass as well as high molecular mass PAHs (Figure 1), but DCM causes delayed de-emulsification of the sample and huge band broadening of the DCM peak during HPLC analysis affect the result significantly. Hence, n-hexane was chosen for the extraction of PAHs.

De-emulsification of chocolate candy samples was carried out by using ethanol in the range of 0.1 to 10 mL. It was found that 0.5 mL of ethanol was sufficient for demulsify the sample when extracted by n-hexane, whereas other solvents and combinations of them delayed the process as presented in Figure 2.

For purification of the sample extract, silica gel column chromatography technique was employed. The column dimension of 450 × 40 mm was used for the purification procedure. In relation to the degree of purification needed, the different amounts of silica gel in the range of 1 to 3 g were used for chromatographic column packing. The column packing was made such that the silica gel was sandwiched between 0.5 g of anhydrous sodium sulfate. The analytical finding shows that the recoveries obtained for each PAH were increased with increasing amount of silica gel up to 3 g. The maximum recoveries were obtained when 3 g of silica gel used for column packing, further increase in the amount of silica gel decreased the recovery percentage of PAHs from chocolate candy samples (Fig. 3). Thus, 3 g silica gel was selected for column packing.

Instrumentation. A HPLC system, equipped with Waters-515 binary pump, UV detector-2487 at 256 nm (Waters, Milford, Mass., U.S.A.), Rheodyne injector (7725), C-18 column (LichroCART 250 × 4 mm, dp: 5 μm), and guard column (LichroCART® 4–4, 5 μm) was used. All standards and processed samples were analyzed for 16 PAHs. A column thermostat (Waters) was used to maintain the column temperature at 27 °C during the analysis. Separation of PAHs was carried out with mobile phase composed of acetonitrile : water (70 : 30, v/v) in isocratic mode at
flow rate of 2 mL min⁻¹ with run time of 20 min. Data acquisition and processing were carried out on Empower® software Build 2154 chromatography manager software.

The GC–MS analysis was performed on an auto system XL gas chromatograph equipped with a Turbo Mass detector (Perkin-Elmer, Singapore Pte. Ltd., Singapore). Analytical separation was achieved on a capillary column DB5-MS (30 m × 0.25 mm i.d., 0.25 μm film thicknesses). The carrier gas was helium (99.999%) at flow rate of 1.5 mL min⁻¹. The sample (1 μL) was injected in a pulsed split less mode. Pulse pressure was 12.64 psi for 0.30 min.

The oven temperature program was 50 °C (1 min) increasing at 25 °C min⁻¹ up to 200 °C, again increasing at 8 °C min⁻¹ up to 316 °C. Total run time was 21.50 min. The injector temperature, 300 °C, transfer line temperature, 280 °C, and the source and quadruple were kept at 300 °C and 180 °C, respectively. The mass spectrometer was operated in full scan mode ranging m/z from 3 to 300 with electron impact ionization energy of 70 eV. Base peak of individual PAH was selected as qualifier ions and other 2 most intense ions in the mass fragmentation of individual PAH were selected as qualifier ions for the identification purpose. All target compounds were identified by GC–MS in the selection ion-monitoring mode using qualifier ion and qualifier ion as shown in Table 1.

### Result and Discussion

The method was optimized by using different solvents for liquid–liquid extraction, by varying volume of de-emulsifying agent and quantity of silica gel for purification successively. The optimized method consisted of 3 g of sample, diluted in 30 mL of de-ionized water, extracted with 5 mL of n-hexane thrice, and de-emulsified with 0.5 mL of ethanol, having extraction time of 10 min each. The extract was dried by nitrogen evaporator and purified by using silica gel column made up of 3 g activated silica gel sandwiched in between 0.5 g of anhydrous sodium sulfate, eluted with the mixture of DCM : n-pentane (2 : 3, v/v). After drying the purified extract under the stream of nitrogen, the residue was dissolved in 0.5 mL acetonitrile and analyzed at wavelength of 256 nm by RP-HPLC.

Quantification of the PAHs present in chocolate candy samples was performed by using an external standard method. To construct the calibration curves, chocolate candy samples spiked with individual PAH at concentrations ranging from 0.01 μg g⁻¹ to 0.05 μg g⁻¹ were prepared and analyzed. Calibration curves showed a linear relationship, between the concentration and peak area, with a correlation coefficient found in the range of 0.9109 to 0.9952 for 16 PAHs. Table 2 shows the values of the correlation coefficients for individual PAHs.

LOD and LOQ were calculated for each PAH and expressed in nanogram per gram. LOD and LOQ were found in the range

### Table 1 – Quantifier ions and qualifier ions used in the selected ion-monitoring of PAHs by gas chromatography-mass spectrometry.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantifier ion</th>
<th>Qualifier ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>128</td>
<td>102, 77</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>152</td>
<td>126, 76</td>
</tr>
<tr>
<td>Acenaphthalene</td>
<td>154</td>
<td>126, 76</td>
</tr>
<tr>
<td>Fluorene</td>
<td>166</td>
<td>139, 115</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>178</td>
<td>152, 89</td>
</tr>
<tr>
<td>Anthracene</td>
<td>178</td>
<td>152, 89</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>202</td>
<td>174, 101</td>
</tr>
<tr>
<td>Pyrene</td>
<td>202</td>
<td>174, 101</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>228</td>
<td>200, 114</td>
</tr>
<tr>
<td>Chrysene</td>
<td>228</td>
<td>202, 114</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>252</td>
<td>224, 126</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>252</td>
<td>224, 126</td>
</tr>
<tr>
<td>Benzo[b]pyrene</td>
<td>252</td>
<td>224, 126</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>276</td>
<td>248, 138</td>
</tr>
<tr>
<td>Dibenzo[a,k]anthracene</td>
<td>278</td>
<td>280, 139</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>276</td>
<td>252, 138</td>
</tr>
</tbody>
</table>

### Table 2 – LOD, LOQ, and correlation coefficient for individual PAH.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LOD (ng g⁻¹)</th>
<th>LOQ (ng g⁻¹)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>1</td>
<td>3.0</td>
<td>0.9199</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>3</td>
<td>9.0</td>
<td>0.9186</td>
</tr>
<tr>
<td>Fluorene</td>
<td>1</td>
<td>3.0</td>
<td>0.9199</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>1.8</td>
<td>5.8</td>
<td>0.9632</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.4</td>
<td>1.2</td>
<td>0.9475</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>0.4</td>
<td>1.2</td>
<td>0.9328</td>
</tr>
<tr>
<td>Pyrene</td>
<td>2</td>
<td>6.5</td>
<td>0.9484</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>0.5</td>
<td>1.8</td>
<td>0.9443</td>
</tr>
<tr>
<td>Chrysene</td>
<td>3</td>
<td>9.0</td>
<td>0.9865</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>0.5</td>
<td>1.5</td>
<td>0.9306</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>1</td>
<td>3.0</td>
<td>0.9275</td>
</tr>
<tr>
<td>Benzo[b]pyrene</td>
<td>0.3</td>
<td>0.9</td>
<td>0.9628</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>1</td>
<td>3.2</td>
<td>0.9194</td>
</tr>
<tr>
<td>Dibenzo[a,k]anthracene</td>
<td>4</td>
<td>12.0</td>
<td>0.9182</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>1</td>
<td>3.0</td>
<td>0.9952</td>
</tr>
</tbody>
</table>

(1) Limit of detection.  
(2) Limit of quantification.

### Figure 3 – Effect of column composition on recovery of each PAH.
of 0.3 to 4 ng g\(^{-1}\) and 0.9 to 12 ng g\(^{-1}\) respectively as shown in Table 2.

Precision of the method was calculated as percent relative standard deviation (RSD%). The chocolate candy samples spiked at 3 concentration levels (4 to 44.8 ng g\(^{-1}\), 8 to 89.6 ng g\(^{-1}\), and 16 to 179.2 ng g\(^{-1}\)) were analyzed 6 times. The value obtained for RSD% were less than 3% for each PAH. Table 3 shows the values obtained of RSD% for individual PAH.

The extraction recoveries were calculated to estimate the extraction efficiency. These were calculated by comparing the amount recovered by the developed method with the total amount initially spiked to the chocolate candy samples. Extraction recovery was expressed as recovery percentage. The values of recovery percentage for individual PAH were shown in Table 3.

The recoveries of the 3 different spiking concentrations (as presented in Table 3) were tested for its significance as per analysis of variance (ANOVA)-two ways. The calculated \(F\) value (10.66) was higher than the \(F\) critical (4.75) value and also the \(P\) value < 0.05, that is, 0.0067, indicating the significance of the recovery of PAHs. The low standard deviation values were in the range of ± 1.86 (4 to 44.8 ng g\(^{-1}\)), ± 3.93 (8 to 89.6 ng g\(^{-1}\)), and ± 6.41 (16 to 179.2 ng g\(^{-1}\)) for all the 16 PAHs in the spiked samples signify the accuracy of the developed method.

A method blank and a solvent blank were analyzed to check the contamination for day-to-day and sample to sample variation. Triplicate injection of 50 \(\mu\)L of PAHs standards was done for each level. The PAHs usually analyzed at 254 nm where the peaks of Flu, BaA, BghiP, and Acc, Chr, Ind were co-eluted out subsequently but by analyzing at 256 nm the separations of co-eluted peaks were observed. Figure 4 shows the chromatogram obtained when the developed method was applied on chocolate candy sample followed by spiking for each PAH in the range of 8 to 89.6 ng g\(^{-1}\).

Application of optimized method—analysis of real candy samples

To establish the effectiveness and the acceptability of the developed method, a variety of chocolate candies were analyzed for the presence of PAHs.

Total PAH contents were found in the range of 2.70 to 235.91 ng g\(^{-1}\) with a mean content of 67.62 ng g\(^{-1}\). In all samples, the low molecular weight PAHs were detected at relatively high concentrations than high molecular weight PAHs in most of the analyzed samples as presented in Figure 5.

Acy was especially prominent in analyzed samples, at a concentration in the range of 3.99 to 183.47 ng g\(^{-1}\). In contrast, Ant, Flu, Py, Ind, and BghiP were detected in few samples. The most carcinogenic PAH, that is, BaP was found in the range of 0.35 to 12.76 ng g\(^{-1}\) with mean content at 1.62 ng g\(^{-1}\). Moreover, in 2 out of 25 candy samples, BaP was detected at relatively high concentrations, the values which even exceeding the EU regulatory control value for BaP (5 \(\mu\)g kg\(^{-1}\)) in cocoa butter. The sum content of all analyzed 16 priority PAHs in chocolate samples, were

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Spk. Conc.(^a) (ng g(^{-1}))</th>
<th>Recovery %</th>
<th>RSD %</th>
<th>Spk. Conc.(^a) (ng g(^{-1}))</th>
<th>Recovery %</th>
<th>RSD %</th>
<th>Spk. Conc.(^a) (ng g(^{-1}))</th>
<th>Recovery %</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nap</td>
<td>96</td>
<td>97.29</td>
<td>0.891</td>
<td>48</td>
<td>74.05</td>
<td>0.758</td>
<td>24</td>
<td>57.38</td>
<td>0.488</td>
</tr>
<tr>
<td>Acy</td>
<td>96</td>
<td>107.02</td>
<td>0.529</td>
<td>8</td>
<td>79.56</td>
<td>1.455</td>
<td>4</td>
<td>23.79</td>
<td>2.492</td>
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<tr>
<td>Flu + Ace</td>
<td>176</td>
<td>103.68</td>
<td>1.011</td>
<td>88</td>
<td>74.65</td>
<td>1.405</td>
<td>44</td>
<td>23.52</td>
<td>1.416</td>
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<tr>
<td>Phe</td>
<td>16</td>
<td>85.82</td>
<td>0.100</td>
<td>8</td>
<td>37.85</td>
<td>0.482</td>
<td>4</td>
<td>22.37</td>
<td>0.968</td>
</tr>
<tr>
<td>Ant</td>
<td>16</td>
<td>89.51</td>
<td>0.178</td>
<td>40</td>
<td>73.09</td>
<td>1.335</td>
<td>20</td>
<td>56.29</td>
<td>1.157</td>
</tr>
<tr>
<td>Flu</td>
<td>44.8</td>
<td>87.28</td>
<td>1.045</td>
<td>22.4</td>
<td>64.07</td>
<td>1.298</td>
<td>11.2</td>
<td>31.99</td>
<td>1.445</td>
</tr>
<tr>
<td>Py</td>
<td>64</td>
<td>99.29</td>
<td>0.680</td>
<td>32</td>
<td>75.81</td>
<td>1.959</td>
<td>16</td>
<td>54.55</td>
<td>4.191</td>
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<tr>
<td>BaA + Chr</td>
<td>80</td>
<td>90.51</td>
<td>0.178</td>
<td>40</td>
<td>73.09</td>
<td>1.335</td>
<td>20</td>
<td>56.29</td>
<td>1.157</td>
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<tr>
<td>BbF</td>
<td>44.8</td>
<td>95.07</td>
<td>0.608</td>
<td>22.4</td>
<td>78.39</td>
<td>3.751</td>
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\(^a\)Spiked concentration.

Figure 4–HPLC chromatogram of PAHs following extraction from chocolate candy sample spiked with 16 PAHs.
Table 4—Concentration of individual PAH in chocolate candy samples expressed as ng g⁻¹ and ND = not detected.

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detected at relatively high concentrations in the present study as compared to previously reported data. The contents of individual PAH expressed in nanogram per gram, found in all analyzed chocolate candy samples are shown in Table 4. The concentrations below the limit of detection were considered as not detected (ND).

Conclusions
The developed method offers an efficient, cost effective, easy sample preparation procedure for the simultaneous determination of 16 PAHs in chocolate candies. The apparatus required for extraction is relatively simple and cheap compared to other sophisticated extraction methods. The method is advantageous in terms of total extraction time, total solvent utilization, as well as the recovery percentage for individual PAH. This method is at par to the conventional method practiced for extraction of PAHs from food matrices.

Correlation coefficient, recovery percentage, and precision were high in this study, which specify the reproducibility and accuracy of the extraction method. Statistical findings bear out that the method is amenable to determine 16 PAHs simultaneously in chocolate candies. Moreover, the results of study imply that PAHs may often be contained in chocolate candies, representing the need to evaluate effective measures to prevent more severe PAHs contamination in chocolate candies in the future.

Acknowledgments
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Determination of polycyclic aromatic hydrocarbons...