

Comparative Evaluation of Different Cell Lysis and Extraction Methods for Studying Benzo(a)pyrene Metabolism in HT-29 Colon Cancer Cell Cultures

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Key Words

Cell lysis • Liquid-liquid extraction • Sonication • Benzo(a)pyrene • HT-29 cells • Colon

Abstract

Lysis and extraction of cells are essential sample processing steps for investigations pertaining to metabolism of xenobiotics in cell culture studies. Of particular importance to these procedures are maintaining high lysis efficiency and analyte integrity as they influence the qualitative and quantitative distribution of drug and toxicant metabolites in the intra- and extracellular milieus. In this study we have compared the efficiency of different procedures viz. homogenization, sonication, bead beating, and molecular grinding resin treatment for disruption of HT-29 colon cells exposed to benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) compound and a suspected colon carcinogen. Also, we have evaluated the efficiency of various procedures for extracting BaP parent compound/metabolites from colon cells and culture media prior to High Performance Liquid Chromatography (HPLC) analyses. The extraction procedures include solid phase extraction, solid-supported liquid- liquid

extraction, liquid-liquid extraction, and homogeneous liquid- liquid extraction. Our findings showed that bead-beating in combination with detergent treatment of cell pellet coupled with liquid-liquid extraction yielded greater concentrations of BaP metabolites compared to the other methods employed. Our method optimization strategy revealed that disruption of HT-29 colon cells by a combination of mechanical and chemical lysis followed by liquid-liquid extraction is efficient and robust enough for analyzing BaP metabolites from cell culture studies.

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Introduction

Benzo(a)pyrene (BaP), a member of the polycyclic aromatic hydrocarbon (PAH) family of compounds is an ubiquitous environmental contaminant to which people are exposed through several routes that include air, water, food, skin contact and occupational settings. Food ingestion is the major route of exposure for a great majority of the population exposed to BaP [1, 2]. Benzo(a)pyrene contamination of food arises from a number of sources, including environment, and food

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processing techniques. Global dietary intake of PAHs range from 0.02 to 3.6 µg/person/day. Foods that contributed to high PAH intake are red meat, dairy products, fats, oils, cereals and vegetables [1]. Epidemiological [3, 4] and laboratory [5] studies have reported a strong association between dietary exposure to BaP and colorectal cancer [3, 4].

Benzo(a)pyrene, subsequent to biotransformation is broken down to a variety of metabolites, some of which are reactive. These metabolites are bound to cellular macromolecules such as proteins, lipids and nucleic acids. While the metabolites released into the culture medium are amenable for liquid-liquid extraction using organic solvents, the metabolites that are present in the intracellular environment require techniques that must disrupt the cell for extraction of these compounds for further processing by High Performance Liquid Chromatography (HPLC) to determine their identity and quantification.

The various methods routinely employed for cell disruption includes homogenization, sonication, bead milling etc. Regardless of the technique used, the ability to extract either parent compound or metabolites when the cells are exposed to low concentrations of BaP present a challenge. This issue becomes more important when data derived from *in vitro* studies could be used for risk assessment purposes [6]. A variety of *in vitro* tools such as subcellular organelles, cell lines, and tissue slices are being used to study the biochemical or molecular mechanisms that underlie gastrointestinal carcinogenesis. The HT-29 cells, originally isolated from colon adenocarcinoma of a Caucasian woman have been used to study cell toxicity [7], chemical-induced differentiation [8], and cellular metabolism [9].

The purpose of this study is three fold; first, it is aimed at determining whether cultured HT-29 colon cells exposed to BaP could metabolically process this toxicant, and release B(a)P metabolites into the culture medium that could be quantified. Secondly, it assesses the efficacy of various cell lysis techniques prior to extracting and quantifying the BaP metabolites in intra- and extra cellular milieus. Finally, our approach also evaluates the efficiency of various extraction methods on the yield of BaP parent compound and metabolites from HT-29 colon cells and culture medium.

Materials and Methods

Preparation of lab ware

To prevent contamination, the following steps were taken. All glassware were acid (15% HNO₃)-washed, followed by a

detergent (Contrad 70, Fisher Scientific, GA) wash and subsequently rinsed with distilled water. The glassware was dried overnight in an oven at 100°C. All lab ware used for cell culture purposes were presterilized.

Cell culture

For the analytical method evaluation conditions, HT-29 cells were purchased from American Type Culture Collection (ATCC; catalog no. HTB-38; Manassas, Virginia). The cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% FBS (Invitrogen) and antibiotics (Penicillin/Streptomycin, Invitrogen). Initially cells were revived by the addition of 1ml of dimethyl sulfoxide (DMSO) stock (HT-29 cell frozen stocks) to 50 ml of media. Every 2 -3 days the media was changed until the cells achieved 80% confluency. After cells were observed to be healthy, stocks were made using 90% FBS and 10% DMSO. Experiments using BaP were conducted as detailed below.

Benzo(a)pyrene treatment

The test chemical BaP (CAS No. 50-32-8; 98% pure; purity confirmed by gas chromatography-mass spectrometry as per the manufacturer) purchased from the Sigma Chemical Co. (St. Louis, MO) was used for preparation of stock solution. Working standard solutions were prepared by appropriate dilution of stock standard solution. The stock and working standard solutions were stored under refrigeration in either amber colored bottles or clear bottles wrapped with aluminum foil to prevent photodegradation of BaP. Because BaP and its metabolites are suspected carcinogens, they were handled in accordance with NIH guidelines for preventing exposure of lab personnel and equipment to this chemical [10].

Cells were seeded onto a 6 well plate or a T75 flask at a density of 20,000 cells/well or 1 X 10⁶ cells respectively. The cells were then synchronized overnight by serum starvation (DMEM/F12 media, 1% FBS) method. HT-29 cells were treated with 10 & 25µM concentrations of BaP in DMSO (0.01%). Duration of HT-29 cell exposure to BaP lasted for 4 days.

The culture conditions were kept at the same level (number of passages, fresh culture medium, actively growing cells, set duration of exposure, predetermined BaP exposure concentration, and same experimenter) to maintain consistency and avoid culture condition-imposed variables on data generation. The experimental procedures adopted are schematically shown in Fig. 1.

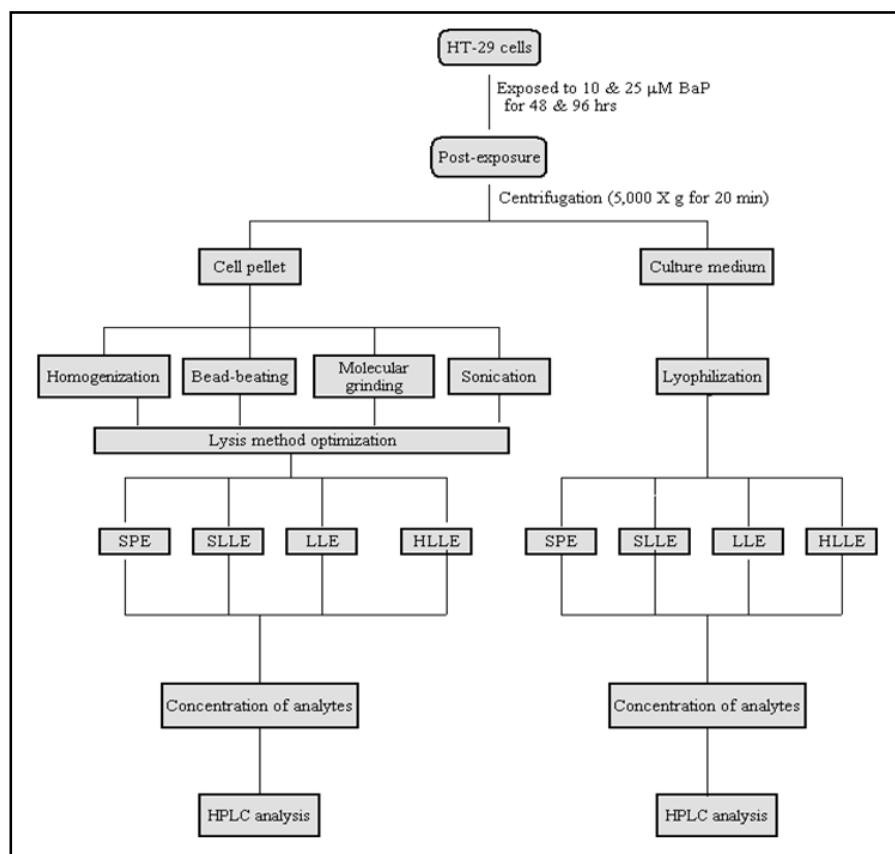
Cell lysis

The following methods were used for cell lysis:

Homogenization. The cell pellet was reconstituted in 2 ml of ice-cold sucrose TKM buffer (sucrose 0.25 M, Tris 80 mM, KCl 25 mM, MgCl₂ 5 mM, pH 7.4) and kept on ice for 5 minutes. A volume of 20 µl of 0.5% SDS was added to the cells, swirled for 2 minutes and broken with a Dounce homogenizer (2 ml capacity; VWR International, Radnor, PA).

Sonication. Cells were subjected to sonication using a VirSonic 50 Ultrasonic Cell Disruptor (SP Scientific Co., Gardiner, NY) operated at 3 cycles of 20s on, 10s off, using a total volume of 1 ml of cell pellet in lysis buffer (G-Biosciences, MO). Care

Fig. 1. Schematic representation of the procedure for extraction of BaP metabolites from colon cells and culture medium. The samples generated from different extraction protocols were analyzed by HPLC. The acronyms used are: SPE: solid-phase extraction; LLE: liquid-liquid extraction; SLLE: solid-supported liquid-liquid extraction; HLLE: homogeneous liquid-liquid extraction; HPLC: high performance liquid chromatography.



was taken to keep the cells on ice during sonication. The disrupted cell suspensions were stored at 4°C until extraction.

Bead-beating. Cell pellet stored in 2 ml of sucrose-TKM buffer and 5% SDS was placed in a polypropylene tube. Spherical, lead-free, acid washed and double distilled water-rinsed soda lime glass beads (0.1 mm diameter) were added to the pellet. The pellet was lysed with a Disruptor Genie (Scientific Industries, New York, USA) at 4°C. Lysates were clarified by centrifugation at 5,000X g for 20 minutes. The particles (beads) settled to the bottom were discarded and the supernatant fraction was removed using a Pasteur pipette and stored at -4°C until extraction.

Molecular grinding resin. Molecular grinding resin (G-Biosciences, Maryland Heights, MO) was added to cell pellet. The resin consists of high tensile micro particles, which effectively disrupt nuclei and other cellular organelles. The pellet was placed in a 1.5 ml microcentrifuge tube and disrupted using a matching pestle affixed to a hand-held homogenizer. After disruption, the contents were subjected to centrifugation. The supernatant fraction was carefully removed and stored at -4°C until extraction.

Lyophilization of culture medium. After separation of cells, the culture medium was lyophilized for 18 h in a VirTis 12EL Freezemobile (SP Scientific Co., Gardiner, New York) and stored at -70°C until used. The lyophilized culture medium was reconstituted in 5 ml of sucrose TKM buffer and subjected to the extraction protocols detailed in Fig. 1.

Liquid-liquid extraction (LLE). The lysates of HT-29 colon cells obtained from the bead-beating method were

transferred to a 10 ml Corning screw cap tube. A volume of 1 ml HPLC grade water was added, followed by 3 ml of methanol and 1.5 ml of chloroform. The tubes were stirred using a vortexer for 60 s and then centrifuged at 5,000 X g for 20 minutes. After centrifugation, the organic and aqueous layers were separated. The organic layer was carefully retrieved into another tube. The tube with the aqueous layer was subjected to a second extraction with water, methanol and chloroform as mentioned above. The organic layer was retrieved again. Organic phases from both extractions were combined and dried under a steady stream of nitrogen N₂. The residue was reconstituted in 300 μl of methanol, passed through Acrodisc filters (0.45 μm; 25 mm diameter; Gelman Sciences, Ann Arbor, MI) to remove particulates. A volume of 30 μl was injected onto HPLC.

The lyophilized culture medium was reconstituted in 1 ml of sucrose-TKM buffer, subjected to LLE as mentioned above. After extraction, the samples were prepared for HPLC analysis.

Homogeneous liquid-liquid extraction (HLLE). An aliquot of 4 mL supernatant solution of sample was placed in a 10 mL screw cap tube with 0.5 mL hexane as extraction solvent and stirred for 1 minute. Then 2 mL of HPLC grade water was added to it. The extraction solvent was separated from the sample via formation of a distinct water-immiscible phase at the top of the vial. The extraction solvent was drawn out by a 1000 μl Hamilton syringe, transferred to a conical tube and evaporated to near dryness under a steady stream of N₂. The residue was reconstituted in 300 μl methanol, filtered to remove particulates and 30 μl of it was injected on to a HPLC column.

Solid phase extraction (SPE). The SPE cartridges (capacity 3.0 ml; particle size 55µm; Waters Corporation, Milford, MA) were affixed to the Supelco Visiprep™ vacuum manifold (Sigma) were used for solid phase extraction. The cartridges were conditioned according to the manufacturer instructions. Next, the cartridges were equilibrated with 3ml of water at a flow rate of 1ml/min. The column was allowed to air dry and analytes were eluted with 3 ml of dichloromethane at a flow rate of 1ml/min. The eluate was collected in glass tubes wrapped with aluminum foil to prevent photodegradation of BaP and/or metabolites. The eluates collected were individually dried under a gentle stream of N₂ at room temperature. The residue was reconstituted in 300µl of methanol, filtered to remove particulates before injecting onto a HPLC column.

Solid supported liquid-liquid extraction (SLLE). Cleanert SLE Plus cartridges containing specially treated diatomite material were purchased from Bonna-Agela Technologies Inc. (Wilmington, DE). They were fixed to the Supelco Visiprep™ vacuum manifold. Samples of lysed cell pellet or reconstituted lyophilized culture media were loaded onto the column by gentle vacuum. After 3 minutes, methanol-chloroform was applied onto the column and the components were let to elute under gentle vacuum. The eluates were collected into glass tubes wrapped with aluminum foil and were individually dried under a gentle stream of N₂ at room temperature. The residue was reconstituted with 500µl of methanol, filtered to remove particulates before injecting onto a HPLC column.

HPLC analysis. The BaP parent compound and metabolites from the cells and culture medium were resolved by a HPLC Model 1200 (Agilent Technologies, Wilmington, DE) equipped with a fluorescence detector. The chromatograph was operated through a ChemStation (Agilent Technologies) for instrument control, data acquisition and analyses. Fifty µl of samples were injected onto a C₁₈ reverse phase column (ODS Pinnacle II, PAH column 4 µm, 250 X 2.1 mm; Restek Corporation, Bellefonte, PA). The column (temperature 33°C) was eluted for 45 minutes at a flow-rate of 1 ml/min with a ternary gradient of water: methanol: ethanol (40: 40: 20%) for 20 minutes, followed by the same gradient at a ratio of 30: 46: 24 for 10 minutes, 100% methanol for 10 min and returning to the initial gradient of 40:40:20 for 5 min. The excitation and emission wavelengths for the detector were 244 and 410 nm respectively. Benzo(a)pyrene metabolite standards were purchased from the National Cancer Institute Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). As BaP and its metabolite standards are potential carcinogens/mutagens, they were handled in accordance with NIH guidelines [10]. Identification of the metabolites was accomplished by comparison of retention times and peak areas of the samples with that of standards.

Recovery experiments for lysis and extraction methods

To the cells, appropriate dilutions of ³H BaP (76 Ci/mmol; GE Healthcare, Piscataway, NJ) was added and incubated for 6 hrs. The radioactivity in the cells and culture medium after incubation were counted. The cells were lysed by the techniques mentioned above. Aliquots of samples prior to- and post lysis were taken for liquid scintillation counting (Beckman Coulter,

| Method | Recovery (%) |
|--------------------|--------------|
| Homogenization | 85 |
| Sonication | 83 |
| Bead-beating | 94 |
| Molecular grinding | 87 |

Table 1. Recovery of spiked ³H [BaP] from cell lysis methods. Appropriate dilutions of ³H [BaP] was added to the cultures and incubated for 6 h. After incubation, cells were lysed as mentioned in “Materials and Methods” section. Radioactivity in samples before and after cell lysis were measured using a liquid scintillation counter.

| Method | Recovery (%) |
|---------------------------------------|--------------|
| Solid phase liquid extraction | 88 |
| Supported liquid extraction | 83 |
| Liquid- liquid extraction | 95 |
| Homogeneous liquid- liquid extraction | 90 |

Table 2. Recovery of spiked ³H [BaP] from cell culture extraction methods. Appropriate dilutions of ³H [BaP] was added to the cultures and incubated for 6 h. After incubation, cells were lysed; cells and media were extracted as mentioned in “Materials and Methods” section. Radioactivity in samples before and after extraction were measured using a liquid scintillation counter.

CA) to compute lysis recovery. Similarly, before extraction, the culture medium was spiked with ³H [BaP]. After extraction, the recovery values were calculated.

Statistics

Every analysis for method optimization was carried out at least three times. A total of 8 samples per method were used. Mean, standard deviation, and standard errors were determined. If relative standard deviation was unusually high, additional measurements were carried out and data outliers were identified by the Grubbs outlier test [11]. The statistical significance of the results (across different treatments) was determined through analyses of variance. The criterion for statistical significance was $p < 0.05$ in all cases. Error bars are given as ± 1 standard error of the mean (SEM).

Results & Discussion

Though sample processing and quantification of toxicants and their metabolites from cell culture systems is not a daunting task as those of whole animal tissues that generate potential interferents, it still is an arduous task owing to the low concentrations of these compounds

Fig. 2. Effect of liquid-liquid extraction (LLE) on BaP metabolite concentrations in culture medium and lysates of HT-29 colon cells ($10^6/\text{ml}$) incubated for 48 & 96 hrs with 10 & 25 μM of benzo(a)pyrene. Post-incubation, cells were lysed by bead-beating treatment; lysates were extracted by LLE and analyzed by HPLC.

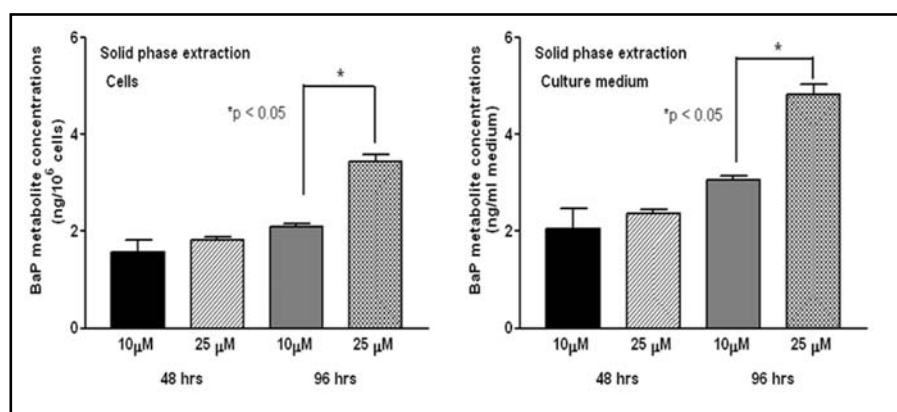
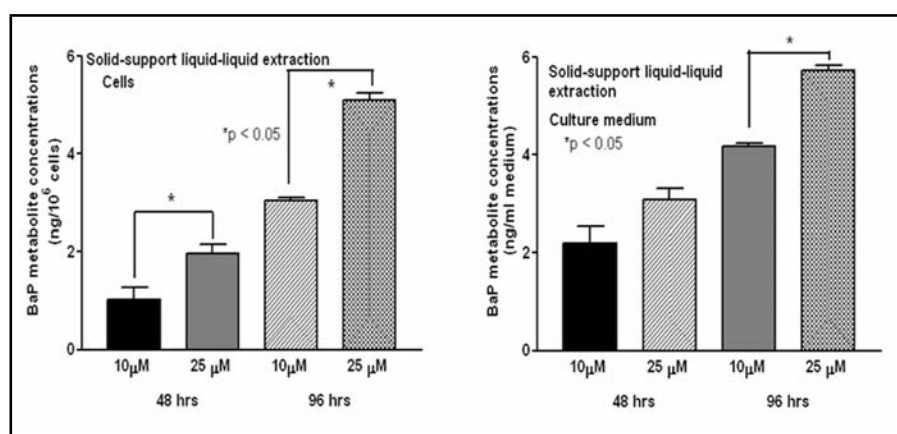


Fig. 3. Effect of homogeneous liquid-liquid extraction (HLLE) on BaP metabolite concentrations in culture medium and lysates of HT-29 colon cells ($10^6/\text{ml}$) incubated for 48 & 96 hrs with 10 & 25 μM of benzo(a)pyrene. Post-incubation, lysed by bead-beating treatment; lysates were extracted by HLLE and analyzed by HPLC.



present in cells or culture media. Therefore, sample processing is a critical step in bioanalysis, which must ensure not only the recovery but also the integrity of sample analytes. Since several methods are used for cell lyses and extraction from normal and human cancerous cell cultures [12], it is necessary to compare/evaluate the extraction efficiency of these methods, and apply the most appropriate method for the quantification of BaP metabolites in colon cell cultures.

As a first step towards cell lysis optimization step, experiments were conducted to select the most appropriate technique (Fig. 1). The cell pellets were subjected individually to homogenization, sonication, bead-beating and molecular resin grinding procedures. The bead-beating of the resuspended pellet coupled with detergent treatment yielded a greater recovery of BaP compared to homogenization, sonication and molecular resin grinding methods (Table 1). The differences in BaP metabolite concentrations were statistically significant ($p < 0.005$; Figs. 2-5).

Though homogenization is one of the most common cell disruption methods [13], the recovery of BaP or its metabolite concentrations in homogenized samples were found to be low in our study. Sonication was advocated as the preferred cell disruption method for increased

product recovery [14]. While the recovery of BaP was high using this method, the metabolite concentrations registered were lower than homogenization. It has been reported that ultra-sonic waves created by the probe in a liquid medium generate tiny gas bubbles that implode (cavitation process) [15]. The temperature and pressure produced as a result of cavitation may have contributed to lysis and disrupted the integrity of metabolites [15]. Both bead-beating and molecular grinding resin methods yielded appreciable recoveries of BaP than homogenization and sonication. The BaP metabolite concentrations were greater than homogenization and sonication. Even though both molecular grinding resin and bead-beating employ micro-particles for cell disruption, bead beating was found to be more effective than molecular grinding resin.

After optimizing the cell lysis method, we next sought to optimize the extraction technique. As part of this strategy we employed different extraction methods to determine the yield of BaP in both cells and culture medium. Liquid-liquid extraction gave the highest recovery of BaP compared to SPE, SLLE, and HLLE. In the order of percent recovery, LLE > HLLE > SPE > SLLE (Table 2). This trend is reflected in BaP parent compound and organic metabolite concentrations as well.

Fig. 4. Effect of solid phase extraction (SPE) on BaP metabolite concentrations in culture medium and lysates of HT-29 colon cells ($10^6/\text{ml}$) incubated for 48 & 96 hrs with 10 & 25 μM of benzo(a)pyrene. Post-incubation, cells were lysed by bead-beating treatment; lysates were extracted by SPE and analyzed by HPLC.

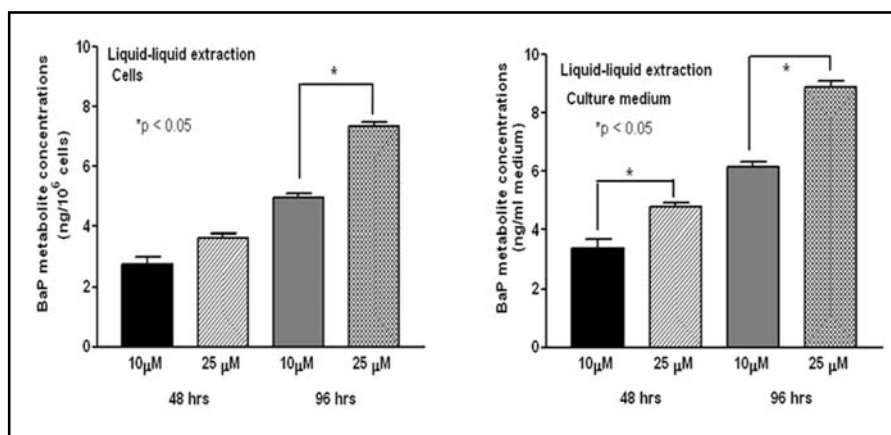
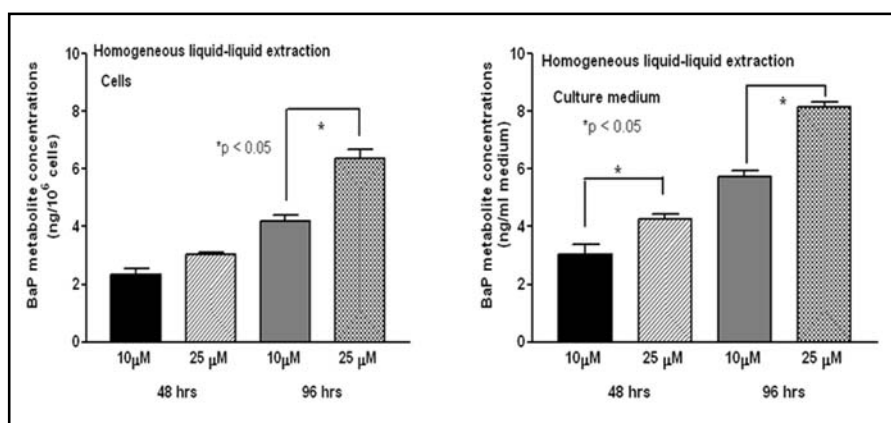


Fig. 5. Effect of solid supported liquid-liquid extraction (SLLE) on BaP metabolite concentrations in in culture medium and lysates of HT-29 colon cells ($10^6/\text{ml}$) incubated for 48 & 96 hrs with 10 & 25 μM of benzo(a)pyrene. Post-incubation, cells were lysed by bead-beating treatment; lysates were extracted by SLLE and analyzed by HPLC.



Both cells and culture media yielded greater concentrations of BaP metabolites when the samples were subjected to LLE (Fig. 2), rather than HLLE (Fig. 3), SPE (Fig. 4) and SLLE (Fig. 5).

Findings of our studies reinforced the notion that liquid-liquid extraction is an effective sample preparation method. This method is based on the principle of differential distribution of analyte between the aqueous medium and an immiscible organic solvent. This process includes thorough and vigorous mixing of both phases and subsequent separation of phases facilitated by mechanical shaking and centrifugation, respectively [16]. Even though several solvent mixtures are used for liquid-liquid extraction purposes, chloroform-methanol mixture (Folch agent) used in our study may have exhaustively extracted BaP and its metabolites. Methanol has been known to denature cell membrane proteins and thereby facilitate the extraction of lipids from inside the cells. As a result, the lipid-bound BaP and/or metabolites were partitioned into chloroform [17]. In addition to lipids, chloroform-methanol can successfully retrieve PAHs from other binding sites such as proteins [18]. It needs to be mentioned that chloroform-methanol mixture has also been found to be effective in extracting BaP and fluoranthene (another

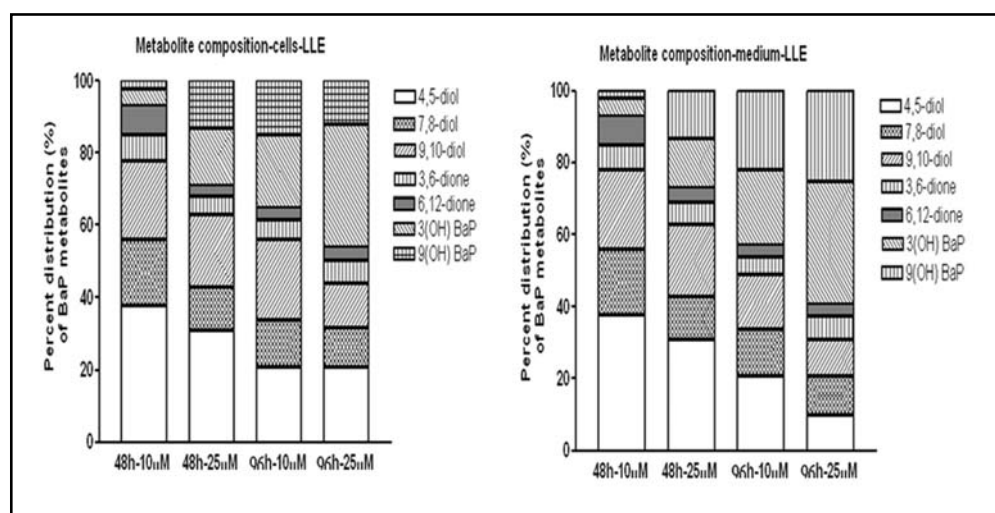
PAH compound) from colon and liver tissues of mice and rats respectively in our laboratory [19-21].

Recently, homogenous liquid-liquid extraction (HLLE) has been touted as an efficient technique for the extraction and determination of PAHs. This method has been employed for detection of PAHs in waste water [22] and vegetables [23]. Unlike LLE, during extraction (phase separation), no interface exists between the water consolute and the extracting solvents. As a result, the surface area of the interface is large enough to hold analytes of interest. The recovery of BaP compound from this method was somewhat lower than LLE.

Next to LLE, SPE provided good recovery for BaP. In recent years, SPE has gained support as an alternative to conventional liquid-liquid extraction due to its reduced solvent use, extraction time, and low costs [24, 25]. In this extraction, BaP is trapped in the C-18 stationary phase because of their chemical nature (non polar) and a stronger affinity for this phase [26]. Using an appropriate solvent, the adsorbed BaP is washed off into an amber tube for further concentration.

We have tested another method, the solid-supported LLE (SLLE), also called as supported liquid extraction (SLE) to examine its efficiency in extracting BaP and/or

Fig. 6. Distribution (percent of total metabolites) of individual BaP metabolite types in culture medium and lysates of HT-29 colon cells (10^6 /ml) incubated for 48 & 96 hrs with 10 & 25 μ M of benzo(a)pyrene. Post-incubation, cells were lysed by bead-beating treatment; lysates were extracted by LLE and analyzed by HPLC.



| Method | Cells | | | | Culture media | | | |
|---------------------------------------|------------|-------|------------|--------|---------------|-------|------------|-------|
| | 10 μ M | | 25 μ M | | 10 μ M | | 25 μ M | |
| | 48 hrs | 96hrs | 48 hrs | 96 hrs | 48 hrs | 96hrs | 48 hrs | 96hrs |
| Solid phase liquid extraction | 0.20 | ND | 0.34 | ND | 0.30 | ND | 0.42 | 0.23 |
| Supported liquid extraction | 0.25 | ND | 0.40 | ND | 0.18 | ND | 0.40 | 0.21 |
| Liquid-liquid extraction | 0.40 | ND | 0.53 | ND | 0.12 | ND | 0.30 | 0.13 |
| Homogeneous liquid- liquid extraction | 0.40 | ND | 0.55 | ND | 0.15 | ND | 0.34 | 0.19 |

Table 3. Concentrations (ng/ 10^6 cells or ng/ml medium) of BaP parent compound (unmetabolized) in cell cultures. Required concentrations of BaP was added to the cultures and incubated for specified time periods. After incubation, cells were lysed; cells and media were extracted as mentioned in “Materials and Methods” section. The sample extracts were concentrated, reconstituted in methanol, filtered to remove particulates and injected onto a reverse phase HPLC equipped with a fluorescence detector for quantification of BaP.

its metabolites. In this method, the cartridges contain an inert medium such as a high-purity diatomaceous earth with a high surface area and high capacity for aqueous adsorption. When the sample is loaded onto the extraction cartridge, the aqueous phase is absorbed while the analytes are spread over the surface of the support in a very thin layer. When a suitable water immiscible organic solvent is applied, liquid-liquid extraction occurs. The high surface area at the interface between the organic and aqueous phases ensures an efficient extraction. This method has been used for routine extraction of PAHs from water samples and human body fluids [27]. However, its usefulness in extraction of PAHs from cell cultures has not been known.

Our studies have revealed that both LLE & HLLE were effective extraction methods compared to SPE & SLLE. While the principle of SPE is based on adsorption of analytes onto a chemically active packing material, LLE operates on the basis of partitioning equilibrium. Probably during extraction, the breakthrough volume of

BaP may have been affected by the concentration of this toxicant in samples and unknown interfering molecules in the packing material.

We also measured the concentrations of different BaP metabolites in cells and culture media to determine whether extraction methods had a bearing on the distribution of BaP metabolite types in these two matrices. Both the cultured HT-29 cells and the culture media registered BaP metabolites. Cultured colon cells were reported to biotransform BaP to an array of metabolites that are egressed into the culture medium [28]. Irrespective of the method used in our study, the total concentration of BaP metabolites were more in culture medium compared to cells. The greater concentrations of BaP metabolites in the medium relative to cultured cells reflect an increased likelihood of metabolic activation of BaP and subsequent excretion into the medium.

Regardless of the extraction methods employed, the following BaP metabolite types were common to all cells and culture media samples tested. The BaP metabolites

were BaP 4,5-diol; BaP 7,8-diol; BaP 9,10-diol; BaP 3,6-dione; BaP 6,12-dione; 3(OH) BaP & 9(OH) BaP. As a representative example, the percent distribution of metabolite types in cells and culture media obtained by LLE is shown in Fig. 6. Neither BaP exposure concentration nor exposure time were found to influence the percent composition of BaP metabolite types in cells and culture media.

There were remarkable differences among the extraction techniques in the relative proportion of BaP metabolite types in cells and culture media. The presence of 7,8-diol and 3,6-; 6,12-quinone metabolites in culture medium suggests the egression of these reactive metabolites from the colon cells into the culture medium. However, compared to the concentration of quinone metabolites, the concentration of BaP 7,8-diol metabolites were low in the medium. Since BaP diol epoxide (BPDE) is unstable in the aqueous medium [29], hydrolysis of this metabolite cannot be ruled out.

For all the extraction techniques used in this study, the presence of unmetabolized BaP in cells was more when exposed to 25 μ M BaP, compared to 10 μ M BaP at 48 h. However, at 96 hrs of exposure to 10 μ M BaP, no parent compound could be detected either in cells or culture media, while at 25 μ M BaP, only culture media registered BaP parent compound (Table 3). The presence of BaP parent compound in cells and culture medium at high BaP exposure concentration could be due to the fact that the capacity of cells to metabolize BaP may have reached a saturation point at 25 μ M BaP exposure concentration. Our results are in agreement with that of other researchers [28, 30, 31] who also detected BaP parent compound in various human cell cultures exposed to BaP concentrations used in this study.

Given the different types of BaP metabolites detected in cell lysates and culture media, a brief account of their generation and relevance to carcinogenesis and toxicity is deemed appropriate. Initial oxidation of BaP is catalyzed by cytochrome P450 (CYP) family of enzymes (CYP1A1, CYP1A2, and CYP1B1), which yield arene oxides (9-OH-BaP, 7-OH-BaP, 6-OH-BaP, 3-OH-BaP, and 1-OH-BaP). These arene oxides either rearrange to phenols or undergo hydration catalyzed by epoxide hydrolase generating BaP-9,10-diol; BaP-7,8-diol; and BaP-4,5-diol [32, 33]. Some of the metabolites such as 7,8-dihydrodiol is activated into 7,8-dihydrodiol 9,10-epoxide, a highly electrophilic chemical species, which binds with cellular nucleophiles such as DNA to cause damage to the cells. Also, during BaP metabolism, some unstable metabolites such as 6-hydroxy-BaP are formed

that undergo autooxidation to generate 1,6-, 3,6- and 6,12-quinone metabolites, which are stable [34]. The radical cations formed as intermediates during BaP metabolism react with water to form quinones and phenols that are highly reactive and interact with DNA to initiate carcinogenesis [35]. The role of the above-mentioned metabolites in carcinogenesis is well known [34]. However, during the conversion of quinones to quinols, reactive oxygen species (ROS) are also produced [36]. Production of ROS leads to severe oxidative stress, eventually contributing to toxicity [37].

The aim of this study was to evaluate the proficiency of various cell lysis and extraction methods and optimize a method for quantifying BaP metabolites from colon cells. Hence we have only focused on providing quantitative and qualitative distribution for BaP organic metabolites only. Subsequent to method optimization, we intend to delineate BaP concentration-response relationship with regard to temporal variations in extracellular and intracellular BaP metabolite levels and types from both organic and aqueous fractions. These aspects together with the activity and expression profiles of BaP biotransformation enzymes will provide a comprehensive picture of BaP metabolism in HT-29 cells and will be the subject of future studies.

In conclusion, our studies revealed that bead-beating coupled liquid-liquid extraction are the ideal cell lysis and extraction methods respectively vis-à-vis other methods for studying BaP metabolism in colon cell cultures. Even though HT-29 cells were employed, the cell lysis and extraction techniques adopted in the present study could be deployed for other PAH-exposed colon tumor cells such as Caco-2, DLD-1, HCT 116, KM12SM, LOVO, LS174T, LT97, SW480, SW620, lung, breast, prostate, ovarian tumor cells, explants of tumors and non-tumor cells such as alveolar cells, aortic endothelial cells, hepatocytes etc.

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Few errors occurred in the article by Myers et al. entitled "Comparative Evaluation of Different Cell Lysis and Extraction Methods for Studying Benzo(a)pyrene Metabolism in HT-29 Colon Cancer Cell Cultures" published in Cell Physiol Biochem 2011; 28(2):209-218.

There is a misplacement of legends for the Figures 2 through 5. The legend printed for Figure 2 goes with Figure 4; Figure 3 goes with Figure 5; Figure 4 goes with Figure 2 and Figure 5 goes with Figure 3.

Legends printed in paper

Fig. 2 (Page 213)
Fig. 3 (Page 213)
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Correct figure the legend corresponds to

Fig. 4 (Page 214)
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