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Liquid chromatography–mass spectrometry analysis of hydroxylated polycyclic aromatic hydrocarbons, formed in a simulator of the human gastrointestinal tract

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Abstract

Described is a liquid chromatography–mass spectrometry (LC–MS) procedure for the determination of hydroxylated biotransformation products of polycyclic aromatic hydrocarbons (PAH) in the human gastrointestinal tract. The formation of hydroxylated PAHs was monitored upon incubation of PAHs with colon microbiota from the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The analytical method consisted of a biomass removal step followed by a solid phase extraction (SPE) step using C18 packed columns to remove non-digested food compounds and microbial metabolites that interfere with the detection of the target compounds. For quantification, 9-hydroxyphenanthrene $^{13}C_6$ was used as the internal standard. The detection limits of the hydroxylated PAHs were generally in the range 0.36–14.09 $\mu g\,l^{-1}$, based on a signal/noise ratio of 3:1. The recovery of hydroxylated PAHs in intestinal suspension was variable ranging from 45 to 107%, with relative standard deviation (R.S.D.) between 5 and 17%. The analytical procedure was used to show the microbial production of 1-hydroxypyrene and 7-hydroxybenzo(*a*)pyrene, metabolites that may give colon incubated PAHs bioactive properties.

Keywords: Polynuclear aromatic hydrocarbons

1. Introduction

The levels of polycyclic aromatic hydrocarbons (PAH) in the environment has remained an area of extensive study in modern times, largely because of their prevalence in the environment and the mutagenic, carcinogenic and estrogenic effects of their metabolites [1–4]. For urban areas in particular, nearby industry and dense traffic may lead to atmospheric deposition of PAHs at levels up to 20 $\mu g\,m^{-2}$ per day [5]. Inhalation of PAH containing particulates and ingestion of contaminated food are important exposure routes to the human body [6,7]. PAH accumulation in the upper soil layers may also pose a serious risk to public health through for example, possible ingestion of contaminated soils or badly cleaned vegetables from these soils. There is growing interest in the study of hydroxylated PAH metabolites, as important intermediates of PAH biotransformation processes in the human body [8]. Monitoring of urinary or biliary 1-hydroxypyrene and other PAH metabolites is often used as biomarkers for PAH exposure in aquatic and terrestrial ecosystems, but also for exposure to humans [9,10]. Apart from their importance as transformation products, hydroxylated PAHs may possess estrogenic properties [4] and some are related to mutagenic and carcinogenic effects.

Analysis of hydroxylated PAHs and PAH metabolites in general is usually performed using high-performance liquid chromatography with fluorescence detection (HPLC-F) [11–13]. Earlier studies of PAH biotransformation have also applied gas chromatography (GC) coupled to mass spectrometry (MS) with chemical ionization (CI) to screen for PAH hydroxylates, while the PAH parent compounds were analyzed through classical HPLC with diode array

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detection (DAD) [14]. The liquid chromatography–mass spectrometry (LC–MS) methods have utilized atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) in both positive and negative ionization mode to screen for hydroxylated PAH compounds [15,16]. However reported detection limits of around 0.5 μg ml^{-1} are relatively high when taking into account that concentrations in the range 0.039–2.5 μg ml^{-1} have been previously used for detecting in vitro mutagenic effects and that urinary 1-hydroxypyrene levels of only 2.5 ng ml^{-1} may occur [17,10].

Thus far, most research on PAH exposure and detection of metabolites has been performed by analyzing biliary or urinary samples. Little is known however, about the biotransformation processes of environmental contaminants by intestinal microbiota from the gastrointestinal tract. This is due in part to a need for analytical methods that are suitable for determining the transformation of xenobiotics compounds in the microbe and enzyme diverse environment of the colon [18]. In this study, we present a LC–ESI–MS methodology for the quantification of hydroxylated PAH metabolites and some selected PAHs in water and the human gastrointestinal tract. The detection of these microbially formed hydroxylated PAHs in intestinal suspension has not been described previously.

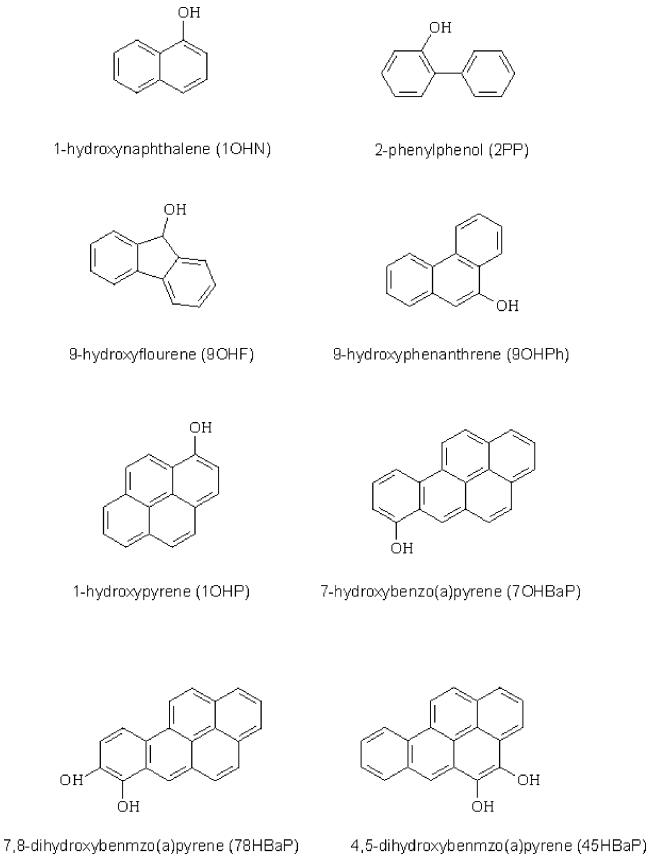

Fig. 1. Chemical structures of PAH hydroxylates.

2. Experimental

2.1. Reagents

The PAH hydroxylates (Fig. 1) investigated were 1-hydroxynaphthalene (1OHN), 1-hydroxypyrene (1OHP), 9-hydroxyfluorene (9OHF) and 9-hydroxyphenanthrene (9OHPh), obtained from Sigma–Aldrich (Bornem, Belgium) and 7-hydroxybenzo(*a*)pyrene (7OHBaP), 7,8-dihydroxybenzo(*a*)pyrene (78OHBaP), and 4,5-dihydroxybenzo(*a*)pyrene (45OHBaP) obtained from the NCI Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, USA). One additional compound, 2-phenylphenol (2PP) (Sigma–Aldrich, Bornem, Belgium), was also investigated as a putative PAH metabolite. PAH parent compounds were obtained from Sigma–Aldrich (Belgium), Janssen Chimica (Geel, Belgium) and Supelco (Oakville, Canada). Stock solutions of the PAH hydroxylates were prepared in methanol and contained 100 μg ml^{-1} 1OHN, 90 μg ml^{-1} 1OHP, 93 μg ml^{-1} OHF, 98 μg ml^{-1} 9OHPh, 124 μg ml^{-1} 2PP, 8 μg ml^{-1} 7OHBaP, 16 μg ml^{-1} 45OHBaP and 11 μg ml^{-1} 78OHBaP. A series of working standards was prepared by diluting the stock solution with 50% methanol to final concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.01 μg ml^{-1} for each individual PAH hydroxylate. In addition, similar standards were prepared with centrifuged colon suspension as diluent to compensate for possible matrix effects in the quantitative LC–ESI–MS analysis of colon suspension samples.

Stock solutions of the corresponding PAH parent compounds were made in acetonitrile with concentrations of 218 μg ml^{-1} naphthalene, 220 μg ml^{-1} pyrene, 212 μg ml^{-1} fluorene, 196 μg ml^{-1} phenanthrene and 164 μg ml^{-1} benzo(*a*)pyrene. To determine the removal of other PAH components during gastrointestinal digestion, an additional set of stock solutions containing 252 μg ml^{-1} acenaphthylene, 180 μg ml^{-1} anthracene, 180 μg ml^{-1} fluoranthene, 164 μg ml^{-1} benzo(*a*)anthracene and 168 μg ml^{-1} chrysene were used. No hydroxylated derivatives from these latter PAHs were analyzed. Mixed standard dilution series were prepared by diluting the stock solution with 50% methanol to final concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.01 μg ml^{-1} for each individual PAH compound. In addition, similar standards were prepared using blank colon suspension as the diluent. These solutions were used for LC–DAD analysis.

2.2. Incubation

Samples were taken from the colon vessels of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [19]. This dynamic model of the human gastrointestinal tract consists of five compartments representing the stomach, duodenum, colon ascendens, colon transversum and colon descendens, respectively

(http://www.avecom.be/shime_i.html). The colon suspension contains an in vitro cultured microbiota that is isolated from human feces and is representative for the in vivo colon microbial ecology [19]. The organic matter content of colon suspension typically varies around 2.2 g l^{-1} and originates from some food compounds but mainly microbiota at concentrations of 11 log CFU ml^{-1}.

A volume of 500 ml of colon suspension was sampled from the SHIME reactor and distributed in aliquots of 50 ml to ten penicillin flasks. Each flask contained a different PAH compound. To avoid solubility problems, PAHs were first dissolved in ethanol and then introduced separately into the respective flasks at final concentrations for naphthalene, 2.56 μg ml^{-1}, acenaphtylene, 3.04 μg ml^{-1}, fluorene, 3.32 μg ml^{-1}, phenanthrene, 3.56 μg ml^{-1}, anthracene, 3.88 μg ml^{-1}, pyrene, 4.04 μg ml^{-1}, fluoranthene, 4.05 μg ml^{-1}, benzo(*a*)anthracene, 4.56 μg ml^{-1}, chrysene, 4.56 μg ml^{-1} or benzo(*a*)pyrene, 5.04 μg ml^{-1}. These suspensions were then incubated for 24 h at 37 °C. After this colon incubation, samples were centrifuged at 3000 × *g* for a duration of 10 min to remove biomass and subsequently stored at −20 °C prior to analysis for PAH hydroxylates and parent compounds.

In order to assess the extent of bacterial degradation, a number of control samples were included in the experimental setup. Firstly, in order to determine whether hydroxylated PAHs could be formed by extracellular enzymes, PAHs were incubated in colon suspension that had been centrifuged (3000 × *g*, 10 min) to remove microbial biomass. Secondly, un-dosed colon samples were analyzed to serve as a negative control as they presumably do not contain any of the PAHs. Thirdly, a stomach and small intestine digest of the PAHs prior to the colon incubation was performed as previously described [20] and analyzed. These digestion steps contained no bacteria, thus, no biotransformed PAHs should be measured from these samples.

Samples were also incubated in the presence of β-glucuronidase and aryl sulfatase, both obtained from Sigma–Adrich (Belgium). After the PAH parent compounds had been incubated in SHIME suspension, a 1 ml aliquot of these samples were diluted in 1 ml 0.1 M acetate buffer and the pH was adjusted to 5 with sodium hydroxide. A volume of 400 μl β-glucuronidase (100 U ml^{-1}) and 250 μl aryl sulfatase (60 U ml^{-1}) were added and the mixture was incubated for 6 h at 37 °C to hydrolyze the PAH conjugates.

2.3. Sample preparation

Due to the complexity of the colon suspension from the SHIME reactor, matrix interference was anticipated. To assess the extent of such interfences, calibration standards were prepared in both MilliQ® (MQ) water and centrifuged colon matrix. Standard curves and recoveries in MQ water and colon matrix were compared to determine the influence of the colon matrix on the detection and quantification of the PAH hydroxylates and parent compounds. All samples were thawed and subsequently subjected to a solid phase extraction (SPE) using PrepSep™ C18 (250 mg) (Fisher Scientific, Edmonton, Canada). The C18 columns were placed on top of a SPE vacuum manifold (Chromatographic Specialty, Ontario, Canada) to aid solvent elution through the column. The C18 packing material was first conditioned with 10 ml of methanol and subsequently rinsed with 10 ml water. Sample volumes of 5 ml were loaded on the columns; the aqueous solution was eluted as waste using a gentle vacuum together with 10 ml of MQ water to remove hydrophilic impurities that were present within the complex colon matrix. For the mixed standard solutions, the analytes (PAHs and hydroxylates) were eluted together with other hydrophobic compounds by loading four times 2.5 ml of methanol on the column. During the method development phase, each eluent was analyzed separately to determined in which fraction a given analyte was recovered. As no significant amounts of analytes eluted in the fourth fraction, all other samples were eluted with 7.5 ml of methanol. Aliquots of 1.0 ml of the sample extracts were subsampled into amber glass vials and stored at 4 °C prior to LC–ESI–MS analysis.

2.4. Instrumental conditions

HPLC analysis was performed using a Waters 2695 (Milford, MA, USA) separation module. The HPLC pump was primed with fresh eluent on a daily basis. The selected column was a 2.1 mm × 100 mm, 3.5 μm particle size, Waters XTerra MS C18 column (Milford, MA, USA) which was kept at a constant temperature of 26 °C. The binary eluent system consisted of methanol:water 90:10 (v/v) (eluent A) and methanol:water 10:90 (v/v) (eluent B). Gradient elution was performed using 50% eluent A for 5 min, then a linear gradient from 50 to 95% eluent A for 30 min at a flow-rate of 200 μl min^{-1}. At the end of each cycle, 95% eluent A conditions were held for 5 min to ensure all sample components were eluted from the column. An injection volume of 10 μl was utilized employing a Waters 2695 autosampler for both samples and calibration standards. A diode array UV detector was plumbed inline prior to the mass spectrometer for detection of the parent PAHs at a wavelength of 280 nm.

Mass spectrometry analysis was performed with a Quattro Ultima Mass spectrometer (Micromass Technologies, Manchester, UK) that was equipped with an electrospray interface operating in the negative ion mode. Instrumental control and data acquisition was performed with MassLynx software version 3.5. The ESI source was operated at 90 °C, desolvation temperature 200 °C, cone voltage 61 V, and a capillary voltage of 2.74 kV. Nitrogen gas served as the cone gas (flow-rate of 159 l h^{-1}), desolvation gas (490 l h^{-1}) and nebulizer gas (set to maximum). The detector multiplier voltage was set to 650 V. Selected ion monitoring (SIM) was employed for quantitative analysis monitoring the $(M-H)^-$ of *m/z* 143.2 for 1OHN, 169.2 for 2PP, 181.2 for 9OHF, 193.2 for 9OHPh, 199.2 for 9-hydroxyphenanthrene $^{13}C_6$ (internal standard), 217.2 for 1OHP, 267.2 for 7OHBaP and

285.2 for 4,5OHBaP and 7,8OHBaP. The dwell-times were set at 0.3 s and the inter-scan delay time was 0.1 s. As electrospray is a soft ionization technique, only the $(M-H)^-$ were formed. As it is preferred to monitor more than one ion per component of interest for quantification and confirmation purposes, cone induced and collision induced dissociation were evaluated. Under various experimental conditions the molecular ions did not form product ions therefore confirmation using a secondary SIM channel or reaction monitoring was not possible. In lieu of qualifier ions, the in-line diode array UV detector was used as a secondary confirmation of the identification of the hydroxylated PAHs. Detection of possible unknown metabolites of the PAH parent compounds was also performed with the mass spectrometer operated in full scan mode over a range of 100–450 *m/z*.

3. Results and discussion

3.1. Calibration and matrix effects

Detection of hydroxylated PAHs in an intestinal suspension has not been described before. The difficulty of analysis of intestinal suspension pertains to the complexity of the matrix. The colon matrix is comprised of non-digested food components, several excretion products and hundreds of microbial metabolites. Following sample clean-up, several hydrophobic compounds may still co-elute with the target analytes and thus interfere with the identification and quantification of the PAH hydroxylates during LC–ESI–MS analysis. In order to compensate for matrix effects on both chromatographic separation and electrospray ionization suppression/enhancement, a centrifuged blank colon suspension (3000 × *g*, 10 min) was used as the diluent when preparing the calibration standards. Calibration curves were therefore independently prepared, using either MilliQ® water or colon suspension that had been spiked with PAH standards and their hydroxylated derivatives at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg l^{-1}. For most of the compounds, linear correlations ($R^2 > 0.99$) between peak areas and analyte concentrations were obtained. The calibration curve for 45OHBaP and 78OHBaP showed a lower correlation ($R^2 = 0.947$), whereas quadratic calibration curves were obtained for pyrene ($R^2 = 0.999$) and fluoranthene ($R^2 = 0.998$).

Under the experimental conditions utilized for the gradient and column, the majority of the analytes were base-line resolved from interfering components, as illustrated in Figs. 2 and 3. Representative ion chromatograms for the hydroxylated PAHs are given in Figs. 2 and 3, respectively, for (a) concentrations of 1 mg l^{-1}, spiked in colon

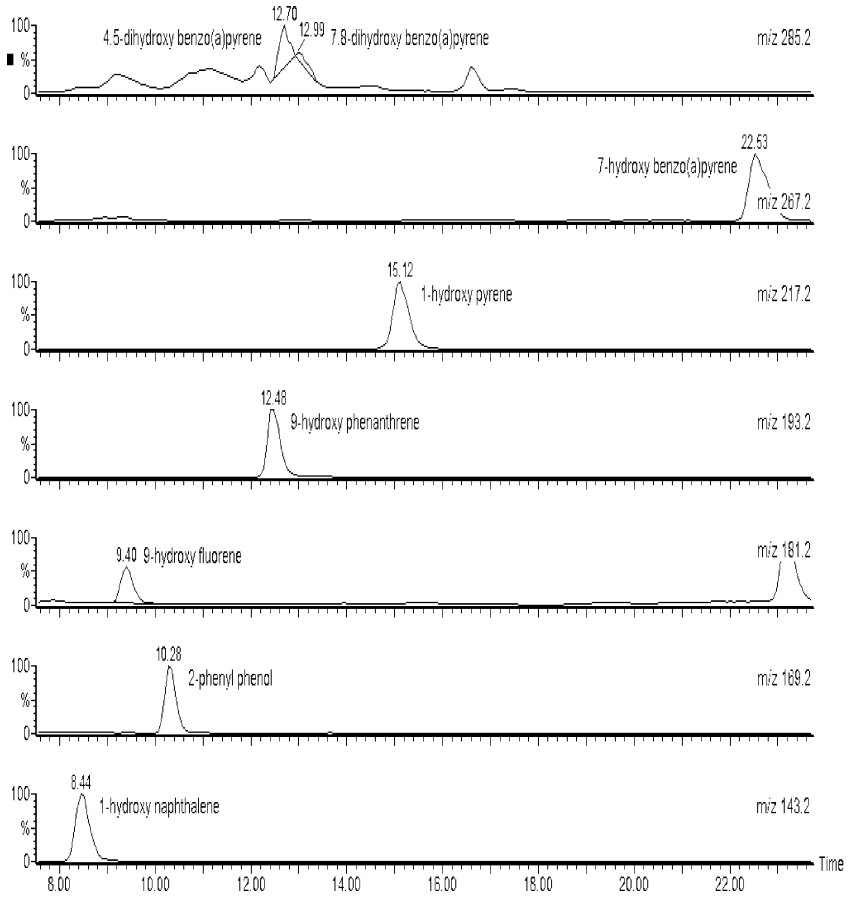

Fig. 2. LC–MS ion chromatogram of the PAH hydroxylates fortified in colon suspension at a 1 mg l^{-1} concentration. Retention time in min.

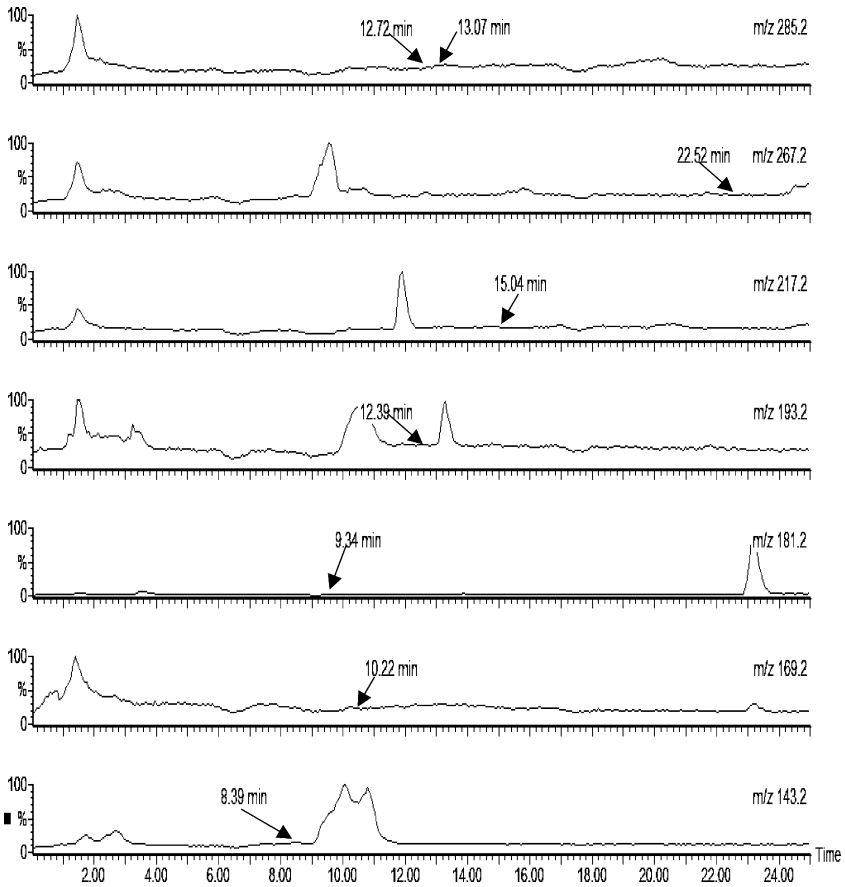

Fig. 3. Chromatogram for *m/z* ratios of PAH hydroxylates in a blank sample, which is typically colon suspension to which no PAHs or hydroxylated PAHs were supplemented. Time is expressed in min. Background signals from the colon suspension matrix eluted at retention times other than those from the standards of the PAH hydroxylates. Hence, little to no matrix interference was expected.

suspension; and (b) blank colon suspension with no spike addition of the target analytes. As shown in Figs. 2 and 3, there was little to no background signals from the colon matrix that interfered with the detection of the selected PAH hydroxylates, nor with the detection of the target PAHs when spiked at 1 mg l^{-1} in colon suspension (Fig. 4). However, the separation of 45OHBaP and 78OHBaP was compromised compared to that obtained from MQ water (data not shown). This loss of chromatographic resolution was probably due to column overloading from the complex sample matrix. The two respective analytes were therefore quantified together as a pair in colon suspensions. Future method modifications should incorporate the improvement of LC separation in order to report individual levels of these two compounds in the complex colon suspension.

3.2. Limits of detection and recovery of analytes

The limits of detection (LOD) for the PAH hydroxylates, based on a signal to noise ratio of 3:1 was 0.26–6.9 μg l^{-1} when determined in MilliQ® water and 0.6–31.3 μg l^{-1} for most hydroxylated PAHs when determined in colon suspension (Table 1). The LODs for 45OHBaP and 78OHBaP in colon suspension were however much higher compared to the other analytes, reflecting residual interference from background signals (Table 1). The limit of quantification (LOQ) for the PAH hydroxylates fall below 50 μg l^{-1} based on the experimental recovery data obtained from the 50 μg l^{-1} fortified SHIME matrix (Table 2) which shows the relative standard deviation (R.S.D.). values for the PAH hydroxylates investigated at this concentration are below 20%. In general, the LODs are higher than those reported for 1OHP, 4 ng l^{-1},

Table 1
Limits of detection (μg l^{-1}) for the PAH hydroxylates

	LOD (water) (μg l^{-1})	LOD (colon) (μg l^{-1})
1-Hydroxynaphthalene	3.5	1.9
2-Phenylphenol	1.4	6.0
9-Hydroxyfluorene	7.0	17.9
9-Hydroxyphenanthrene	1.0	2.1
1-Hydroxypyrene	0.2	0.6
7-Hydroxybenzo(*a*)pyrene	1.2	4.0
4,5-Dihydroxybenzo(*a*)pyrene	3.6	31.3
7,8-Dihydroxybenzo(*a*)pyrene	2.7	21.5

Injection volume was 10 μl for each standard.

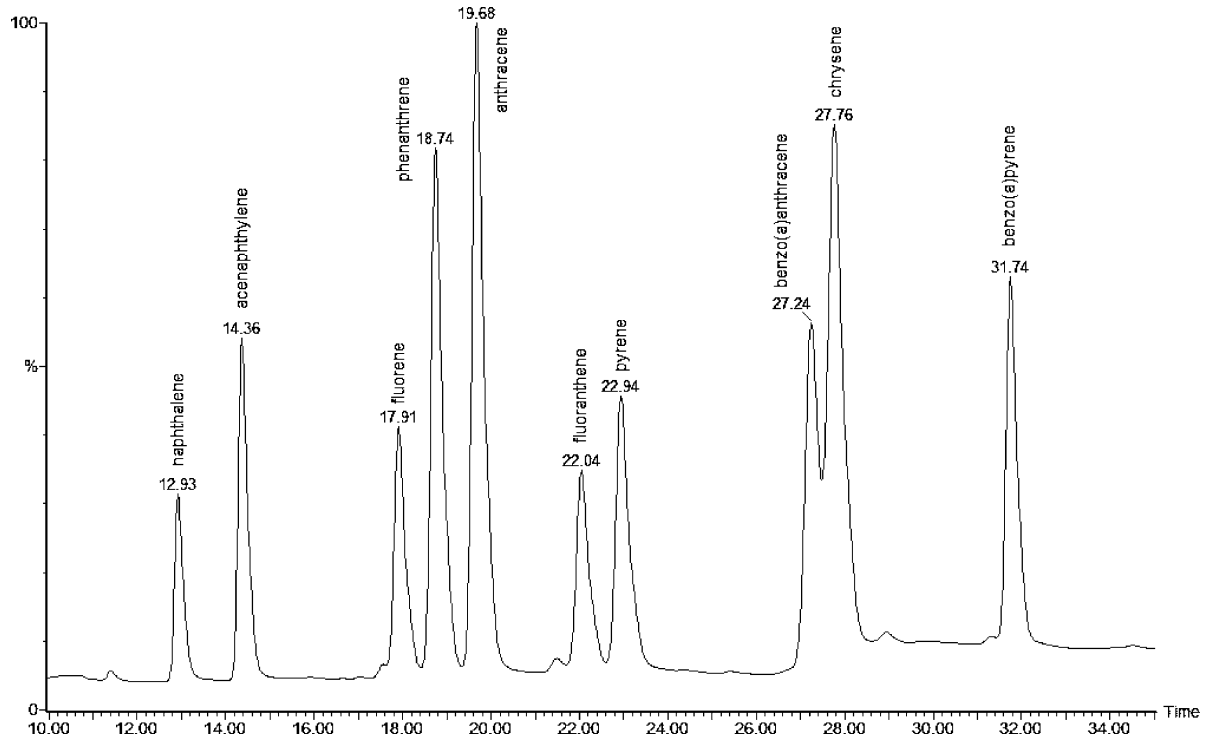

Fig. 4. Retention times of the parent PAH compounds spiked at 1 mg l^{-1} in colon suspension as analyzed by HPLC followed by diode array detection. Retention time in min.

and 3OH BaP, 51 ng l^{-1}, in urine samples [21]. Also for urine samples, Chetiyanukornkul et al. [22] reported a LOD of 0.1 μg l^{-1} for 1OHP, compared to the value of 0.6 μg l^{-1} observed for the colon suspension in the current investigation. However, the colon suspension can be considered as a more complex matrix with more hydrophobic microbial metabolites, compared to the hydrophilic properties of urinary metabolites. Galceran and Moyano [16], who also used an LC–MS method for PAH hydroxylate analysis, arrived at higher LOD values of 500 μg l^{-1}. Considering the LOD from these related studies and the matrix complexity from this study, the presented LC–ESI–MS method offers comparable or improved detection of the PAH hydroxylates in colon suspensions.

The recovery of the PAH hydroxylates was determined in MilliQ® water (clean matrix) at 0.5 mg l^{-1} and in centrifuged colon suspension (complex matrix) at two concentrations, 0.5 and 0.05 mg l^{-1}. Table 2 shows that the recoveries from the MQ water matrix were quite good with values between 82 and 94%. These values are comparable to the recovery values of 80–91% for 1OHP from human urine samples [23,24]. The relative standard deviation values for within-day precision studies ($n = 3$) ranged from 1 to 8%. Only 7OHBaP had a lower recovery percentage of 58%, but the low standard deviation of 4.2% indicates that the results were reproducible. The lower recovery suggests that the solid phase extraction method with methanol as eluting solvent is less selective for the more hydrophobic hydroxylates. The recovery of the pair of dihydroxylates of benzo(*a*)pyrene gave a high recovery of 121%, reflecting uncertainties associated with calibration and recovery of these two analytes.

Table 2
Recovery percentages of PAH hydroxylates fortified in MQ water and SHIME matrix (fortification concentration in parenthesis) ($n = 3$)

	Recovery (%) in MQ water ± S.D. (0.5 mg l^{-1})	R.S.D. (%)	Recovery (%) in SHIME matrix ± S.D. (0.5 mg l^{-1})	R.S.D. (%)	Recovery (%) in SHIME matrix ± S.D. (0.05 mg l^{-1})	R.S.D. (%)
1-Hydroxynaphthalene	94 ± 5.6	6	63 ± 3.1	5	67 ± 3.1	5
2-Phenylphenol	91 ± 7.0	8	61 ± 3.2	5	56 ± 8.0	14
9-Hydroxyfluorene	87 ± 6.9	8	55 ± 6.1	11	45 ± 7.6	17
9-Hydroxyphenanthrene	82 ± 6.2	7	89 ± 6.1	7	83 ± 9.3	11
1-Hydroxypyrene	94 ± 5.6	6	72 ± 4.3	6	57 ± 5.8	10
7-Hydroxybenzo(*a*)pyrene	58 ± 4.2	7	87 ± 12.2	14	74 ± 6.8	9
4,5- and 7,8-Dihydroxybenzo(*a*)pyrene	121 ± 1.8	1	107 ± 13.4	12	83 ± 7.0	8

For the colon matrix containing 0.5 mg l^{-1} PAH hydroxylates, recoveries ranging from 55 to 89% were obtained. Again, the two dihydroxylates of benzo(*a*)pyrene were calculated as a non-resolved pair, leading to a recovery of 107%. At lower concentrations of 0.05 mg l^{-1}, the PAH hydroxylate recoveries ranged between 56 and 83% with one lower recovery of 45% for 9OHF (Table 2). The lower recoveries of the monohydroxylates, especially at lower concentrations may be explained by the complexity of the colon matrix itself, which consists of a wide variety of hydrophobic compounds that are not fully removed during sample clean-up. These components easily bind to the packing material of the C18 columns and thus compete with the PAH hydroxylate or parent compounds investigated. Both at concentrations of 0.5 and 0.05 mg l^{-1}, the more hydrophilic PAH hydroxylates—1OHN, 2PP and 9OHF—appear to have lower recoveries than the more hydrophobic compounds (Table 2). It is therefore possible that some loss of the more hydrophylic compounds occurred when the extraction column was rinsed with MQ water after sample loading. Likewise, the loading capacity may have been compromised by the complex nature of the colon suspensions. Further refinement of the procedure may thus require the use of less hydrophobic column material such as C18 packing material. Despite the general low recoveries, however, the reproducibility was acceptable at 0.5 mg l^{-1}. The within-day precision R.S.D. value ($n = 3$) ranged from 5 to 11%, showing moderate reproducibility. For example, the within-day precision ($n = 3$) showed a good reproducibility with R.S.D. values between 5 and 11% values for 1OHN, 2PP and 9OHF. At a concentration of 0.05 mg l^{-1}, the reproducibility was good with R.S.D. values between 5 and 11%. For 2PP and 9OHF, the reproducibility was lower with R.S.D. values of 14 and 17% at 0.05 mg l^{-1}. These numbers indicate the usefulness of the presented method to quantify PAH hydroxylates in the intestinal suspension.

The corresponding recoveries for the parent PAHs are given in Table 3. For 0.75 mg l^{-1} fortified MQ water, the recoveries were between 100 and 43% with R.S.D. values ranging from 1 to 13.9%. For the more complex SHIME matrix, as expected the recovery was generally lower than those measured for the relatively clean MQ water. Recovery values (obtained from two fortification levels) ranged from 39 to 60% were obtained for the least hydrophobic PAHs, whereas values of 19–42% were obtained for the most hydrophobic PAHs, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene and benzo(*a*)pyrene. The R.S.D. values (1.1–15.2%) ($n = 3$) indicate good to moderate reproducibility. Besides quantifying PAH hydroxylates in the intestinal suspension, the presented method can thus also be applied for quantification of their respective parent PAHs.

3.3. Application of method for the analysis of hydroxylated PAHs in intestinal suspensions

The proposed method was applied to analyze colon suspension in which PAHs at a 5 mg l^{-1} concentration had been incubated for 24 h at 37 °C. This suspension contained a complex microbial community that was comparable to in vivo colon conditions [19]. No hydroxylated PAHs were recovered from centrifuged colon suspension from which the majority of microorganisms was removed. This showed that no extracellular enzymes were involved in the formation of hydroxylated PAHs. As indicated in Fig. 3, no hydroxylated PAHs were measured in un-dosed colon samples which served as a negative control. PAHs that had been incubated in a stomach and small intestinal digestion did not lead to a detection of hydroxylated PAHs either.

In contrast to the negative controls, one of the eight target PAH hydroxylates was detected after 24 h of incubation in colon suspension, namely 1OHP at a concentration of 2.5 μg l^{-1} (Fig. 5). A small peak was observed for 9OHF, however below the limit of quantification. After incubation of a 1 m aliquot of the sample in glucuronidase and aryl sulfatase, a concentration of 4.4 μg l^{-1} was obtained for 1OHP, suggesting that conjugated metabolites had also been formed. Some of the other PAH hydroxylates investigated were also found but at trace level. 7OHBaP was found at a 1.9 μg l^{-1} concentration, whereas shoulder peaks corresponding to the retention time and *m*/*z* ratios for 1OHN and

Table 3
Recovery percentages of PAHs fortified in MQ water and SHIME matrix (fortification concentration in parenthesis) ($n = 3$)

	Recovery (%) in MQ water ± S.D. (0.75 mg l^{-1})	R.S.D. (%)	Recovery (%) in SHIME matrix ± S.D. (1.0 mg l^{-1})	R.S.D. (%)	Recovery (%) in SHIME matrix ± S.D. (0.10 mg l^{-1})	R.S.D. (%)
Naphthalene	108 ± 2.1	3	47 ± 3.6	7.7	49 ± 1.7	4.0
Acenaphthylene	94 ± 3.0	3.2	60 ± 3.8	6.3	50 ± 2.7	5.1
Fluorene	87 ± 1.0	1.2	43 ± 1.1	2.5	51 ± 1.5	3.3
Phenanthrene	91 ± 2.3	2.5	42 ± 0.6	1.3	49 ± 1.2	2.4
Anthracene	79 ± 9.3	11.7	39 ± 0.4	1.1	46 ± 2.5	6.0
Fluoranthene	114 ± 2.5	2.2	41 ± 1.0	2.4	42 ± 3.6	9.3
Pyrene	107 ± 1.1	1.0	40 ± 5.1	1.3	38 ± 2.5	7.1
Benzo(*a*)anthracene	55 ± 7.6	13.9	19 ± 2.1	11.3	23 ± 3.5	15.2
Chrysene	78 ± 7.3	9.3	26 ± 0.1	4.4	30 ± 1.1	4.5
Benzo(*a*)pyrene	70 ± 9.3	13.3	31 ± 1.9	6.0	40 ± 2.9	7.0

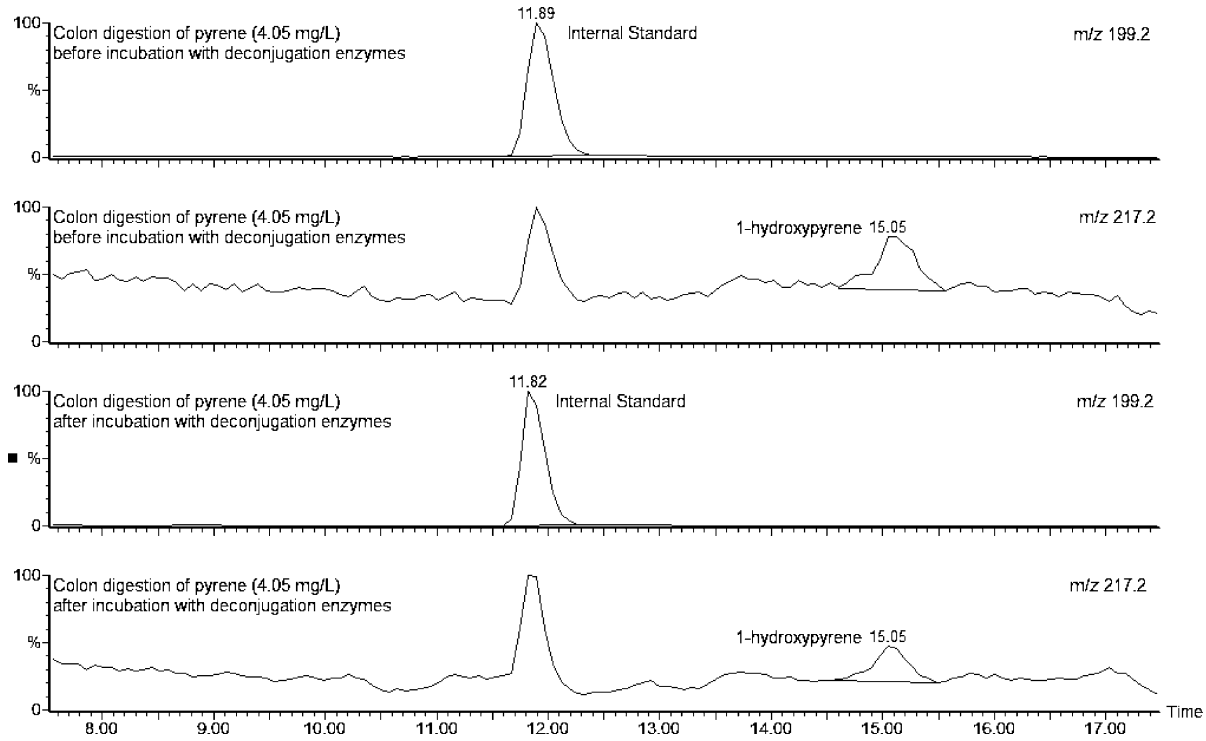

Fig. 5. Chromatogram for internal standards and 1-hydroxypyrene in colon digests of pyrene at a concentration of 4.05 mg l^{-1}: before (upper two chromatograms) and after (lower two chromatograms) incubation with β-glucuronidase and aryl sulfatase as deconjugation enzymes. The *m/z* 217.2 peak at retention times of the internal standard probably is a water adduct $M_{I.S.} + 18$ (199.2 + 18 = 217.2).

9OHF were observed but not quantified as they were below the detection limit.

4. Conclusion

In general, the LC–ESI–MS procedure provided relatively low detection limits for PAH hydroxylates in colon suspensions. Low recoveries were observed for some analytes with overall good precision for the analytes investigated. The practical application of the method for study of complex colon suspensions was demonstrated to reveal that PAHs may be transformed by colon microbiota to hydroxyl derivatives.

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