Isolation and identification of fluoranthene biodegradation products

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Analytical procedures for isolating and identifying the biodegradation products of fluoranthene, a model four ring polycyclic aromatic hydrocarbon, were improved for *ex-situ* experiments using the pure bacterial strain *Pasteurella* sp. IFA. The stable degradation products were determined over a 10 day incubation period. The most efficient method for isolating the metabolites was a two step liquid–liquid extraction using ethyl acetate. Because of the polar nature of fluoranthene metabolites, separation by GC proved inefficient. Their chromatographic properties, however, were improved by derivatisation: silylation with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide for hydroxy and carboxy groups, oxime formation with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride for carbonyl groups, or a combination of both. The metabolites were identified using gas chromatography with mass selective detection (GC-MS/SCAN, SIM). Using this method we were able to identify and quantify six stable fluoranthene metabolites (9-fluorenone-1-carboxylic acid, 9-fluorenone, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid).

Introduction

Fluoranthene, among the most ubiquitous and abundant of the pyrogenic polycyclic aromatic hydrocarbon (PAH) pollutants, is frequently chosen as a model compound for *ex-situ* biodegradation studies. Mixed bacterial cultures[1–5] and selected pure strains, such as *Alcaligenes denitrificans*[2] and *Mycobacterium* sp. BB1 and PYR-1,[3,6,7], are able to mineralise fluoranthene. Using the pure bacterial strains *Pseudomonas paucimobilis*[8] and *Mycobacterium* sp. PYR-1,[3] the identification of a stable fluoranthene biodegradation product was reported for the first time. More recently, ten metabolites were isolated from ethyl acetate extracts of the culture medium *Mycobacterium* sp. PYR-1 by TLC and HPLC, and identified using UV spectroscopy, TLC, GC-MS and NMR.[7] For GC-MS analysis, the acid extractable residues were derivatised by acetylation with acetic anhydride, pyridine or by methylation with diazomethane.[6,9]

For our study of higher molecular weight PAHs using *Pasteurella* sp. IFA, we also chose fluoranthene as the model PAH. The aim of our study was to avoid the use of numerous techniques (IR, UV spectroscopy, HPLC, GC, NMR)[7] and to develop an analytical procedure for determining polar metabolites that can be applied to one general technique such as GC-MS. In our initial study, we could only identify one stable metabolite, 9-fluorenone-1-carboxylic acid;[10] however, in a follow up study using a much improved analytical procedure, we succeeded in determining six metabolites: 9-fluorenone-1-carboxylic acid, 9-fluorenone, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid. This increase in information about the metabolites allowed us to propose a biodegradation pathway of fluoranthene for this bacterial strain.[11,12] In this paper, we discuss in full our improved analytical procedure for isolating and identifying trace amounts of stable fluoranthene metabolic products.

Experimental

Samples

Biodegradation of fluoranthene (20 mg l^{-1}) by *Pasteurella* sp. IFA in aqueous media was performed under controlled conditions.[10] Even though fluoranthene solubility in water is limited (0.20–0.26 mg l^{-1}), an excess fluoranthene source is demanded when working with biotic systems where bacteria constantly remove fluoranthene from the environment. The excess fluoranthene remained in the form of small flakes suspended in the solution. The preparation of the substrate, mineral media with trace elements and the inoculum is described in full elsewhere.[9] All the cultures were incubated at room temperature (22–26 °C) for 10 days.

Analytical procedure

For fluoranthene metabolite isolation, each culture was extracted three times with equal volumes of ethyl acetate (EtAc, 50 ml). The extracts were combined to give the neutral extract. The cultures were then acidified to pH 2.5 using H_2SO_4 and further extracted with another three volumes of ethyl acetate, which when combined gave the acidic extract. Both were dried (anhydrous Na_2SO_4), reduced in volume to 1 ml (N_2), and analysed using a Hewlett Packard (Waldbronn, Germany) 6890-5972 [gas chromatography with mass selective detection (GC-MSD)], fitted with an HP-MS5 crosslinked 5% phenylmethyl silicone capillary column (30 m × 0.25 mm id, 0.25 mm film thickness). Authentic compounds (Aldrich Ch. Co., Milwaukee, WI, USA) of expected fluoranthene metabolites (9-fluorenone-1-carboxylic acid, 9-fluorenone, 9-hydroxyfluorene, 9-hydroxy-1-fluorene-carboxylic acid, adipic acid, phthalic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid) were dissolved in organic solvents and screened by GC-MS.

Direct GC-MS analysis of biodegraded samples

All MS data were obtained by quadrupole MS operating in the SCAN and SIM modes. The sample was ionised using electron impact (EI) with an ion source temperature of 240 °C and an electron energy of 70 eV. Fluoranthene metabolic products in neutral and acidic extracts of each biodegraded sample were initially identified using GC-MS/SCAN (scan interval: *m/z* = 50–500; mass range: ±0.10 amu) and matching the chromato-

graphic retention times and mass spectra with those of authentic compounds. Full mass spectra scan (SCAN) detection was not sufficiently sensitive to detect any metabolites, even after increasing the concentration of the metabolites by combining the incubation sample extracts and removing the undegraded fluoranthene. In the SIM mode, *Rt* ± 0.25 was set for each derivatised compound and the M^+ and base (100%) ions (Table 1) were chosen as the confirming ions. If the base ion (100%) was non-specific, such as *m/z* = 181, or if M^+ was not present, or both, other characteristic ions were chosen. Confirming ions for derivatised metabolites are shown in bold in Table 1. By direct GC-MS/SIM only one metabolite (9-fluorenone) was confirmed in the neutral extract, and two metabolites (9-fluorenone-1-carboxylic acid and 9-fluorenone, Table 2) in the acidic extract.

Derivatisation

To make fluoranthene metabolites (typically containing R–OH, R–COOH, R–C=O, R–CHO groups) more amenable to gas chromatography, we transformed them into non-polar, inert compounds by silylation and oxime formation.

Derivatisation of metabolites containing R–OH and R–COOH groups was performed by silylation with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, Aldrich Ch. Co., Milwaukee, WI, USA).[13–15] This involved adding 50 μl of MSTFA derivatisation agent to 200 μl of standard metabolite solution (0.2 mg ml^{-1}) in a small sealed reaction vial. This was left for 12 h at room temperature with constant agitation to allow the TMS ethers and esters to form.

Authentic standards of suspected metabolites (1 mg) containing carbonyl groups (R–C=O and R–CHO)[16] were derivatised with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA.HCl, Fluka Chemie AG, Buchs, Switzerland). The pH of the metabolite extract was adjusted to 7.4 using dilute HCl and 1.5 ml 0.1 M TRIS buffer (pH 7.4), after which 3 ml of 0.15 M KCl and 1 ml of 0.05 M PFBHA.HCl were added. The sample was stirred for 3 h at room temperature. Formed pentafluorobenzyloximes (PFBO) were extracted three times with a mixture of cyclohexane and diethyl ether (4:1, Merck KGaA, Darmstadt, Germany). The solvents were removed by N_2 and the PFBO products were dissolved in 100 μl of toluene.

Each extract was first derivatised with PFBHA.HCl and divided into two halves. One half of each derivatised sample was analysed by GC-MS, and the remainder was derivatised with MSTFA so that all the samples had been derivatised with both derivatising agents. Fig. 1 shows the analytical scheme for the sample preparation of fluoranthene PFBO-TMS derivatives.

Table 1 Characteristic fragment ions of MSTFA, PFBO and PFBO-TMS fluoranthene derivatives (selected SIM mode ions are shown in bold)

Metabolites	Fragment ions (*m/z*)
MSTFA fluorenone-1-carboxylic acid	**281** (100%), 151, 207, 179, **296** (M^+)
MSTFA 9-hydroxyfluorene	165 (100%), **254** (M^+), **239**
MSTFA 9-hydroxy-1-fluorene-carboxylic acid	207 (100%), **355**, 179, 280, **370** (M^+)
MSTFA adipic acid	73 (100%), 75, 111, 147, **275**, 129, **290** (M^+)
MSTFA phthalic acid	147 (100%), **295**, 73, 221, **310** (M^+)
MSTFA 2-carboxybenzaldehyde	133 (100%), 105, 179, **207**, 77, **222** (M^+)
MSTFA benzoic acid	**179** (100%), 105, 77, **194** (M^+)
MSTFA phenylacetic acid	73 (100%), 164, **193**, 91, 118, **208** (M^+)
PFBO fluorenone-1-carboxylic acid	181 (100%), **419** (M^+), 375, **238**
PFBO 9-fluorenone	**375** (M^+, 100%), 181, 164, **194**, 345
PFBO 2-carboxybenzaldehyde	181 (100%), 148, 134, **300**, **345** (M^+)
PFBO-TMS fluorenone-1-carboxylic acid	181 (100%), 280, **491** (M^+), **401**, 476
PFBO-TMS 2-carboxybenzaldehyde	181 (100%). 220, 130, **402**, **417** (M^+), 328

Table 2 Metabolite identification results using GC-MS/SCAN, SIM

	GC-MS/SCAN	GC-MS/SIM
Neutral extract	u.d.l.[a]	9-Fluorenone
Acidic extract	u.d.l.	9-Fluorenone-1-carboxylic acid, 9-fluorenone

[a] u.d.l. = under detection limit.

GC-MS analyses of derivatised biodegraded samples

All neutral and acidic extracts were derivatised in the same order as the authentic standard compounds so that the presence of all possible metabolites was covered.[10] Initially, the authentic metabolites of fluoranthene (except metabolite 9-fluorenone) were derivatised using MSTFA. The resultant TMS ethers and esters produced a typical mass fragmentation pattern with an intense ion fragment at M − 15, corresponding to the loss of a $–CH_3$ group from the molecular ion. The ions at *m/z* = 73 $[(CH_3)_3Si]^+$, *m/z* = 75 $[HO{=}Si(CH_3)_2]^+$ and *m/z* = 147 $[(CH_3)_2Si{=}O{–}Si(CH_3)_3]^+$ are characteristic for MSTFA derivatisation. The ion at *m/z* = 73 is usually the base ion. Also obvious is the $[M - 89]^+$ or $[M - 90]^+$ ion formed by the cleavage of the TMS–O or TMS–OH group if suitable H is available for rearrangement. GC-MS of the PFBO derivatives reveals intense ions at *m/z* = 181 $[F_5(C_6H_5CH_2)^+]$, $[M - 181]^+$ and $[M - 197]^+$. In the last fragment ion, besides $F_5(C_6H_5CH_2)^+$ oxygen is eliminated. The fragment *m/z* = 181 is usually the base ion in the PFBO spectrum. Table 1 lists the most characteristic fragment ions of MSTFA and PFBO fluoranthene derivatives. A spectrum showing the characteristic fragmentation of metabolite 2-carboxybenzaldehyde in its underivatised (a) and derivatised form as TMS ester (b) and PFBO-TMS ester (c) is shown in Fig. 2.

Of all the tested metabolites, 9-fluorenone was derivatised only with PFBHA.HCl. The other metabolites, 9-fluorenone-1-carboxylic acid, 9-hydroxyfluorene, 9-hydroxy-1-fluorene-carboxylic acid, adipic acid, phthalic acid, 2-carboxybenzalde-

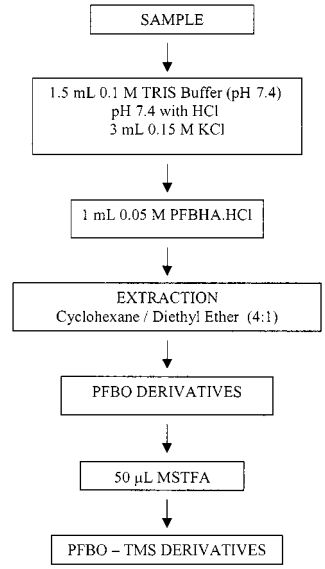

Fig. 1 Scheme of the analytical procedure for the derivatisation of fluoranthene biodegradation products.

hyde, benzoic acid and phenylacetic acid, were derivatised with MSTFA. Metabolites 9-fluorenone-1-carboxylic acid and 2-carboxybenzaldehyde with both types of functional groups (R–C=O, R–CHO) were derivatised using both PFBHA.HCl and MSTFA, resulting in PFBO-TMS ethers and esters.

Quantitative determination of fluoranthene metabolites by GC-MS was achieved using external standards. Since one external standard could not cover the whole range of physico-chemical parameters of the expected metabolites (1–4 ring PAH), two external standards (9-fluorenone-1-carboxylic acid and 2-carboxybenzaldehyde) were applied in our study. The same compounds in derivatised forms were used as external standards for the derivatised metabolic products. External standards were prepared before and after derivatisation at a concentration of 1 mg ml^{-1} and were diluted 2, 5, 10 and 100 times. One microlitre of prepared standard was injected. Triplicate analysis was used to obtain a five point standard calibration curve for both external standards before and after derivatisation.

The purpose of our work was mainly to estimate the amount of fluoranthene that was biodegraded and to identify new metabolites. Due to time limitation and time consuming extraction and derivatisation procedures, samples were run in duplicate. After the remaining fluoranthene had been removed (first step extraction with EtAc) and an extraction efficiency had been applied to each metabolite before and after derivatisation, the obtained metabolite integration peak areas were interpolated from the calibration curves. Extraction efficiencies were determined on the basis of the extraction of known amounts of all authentic compounds and were determined to be between 63% (fluorenone-1-carboxylic acid) and 92% (phenylacetic acid). This procedure provides the mass (μg) of metabolite (pure or derivatised) remaining in the sample.

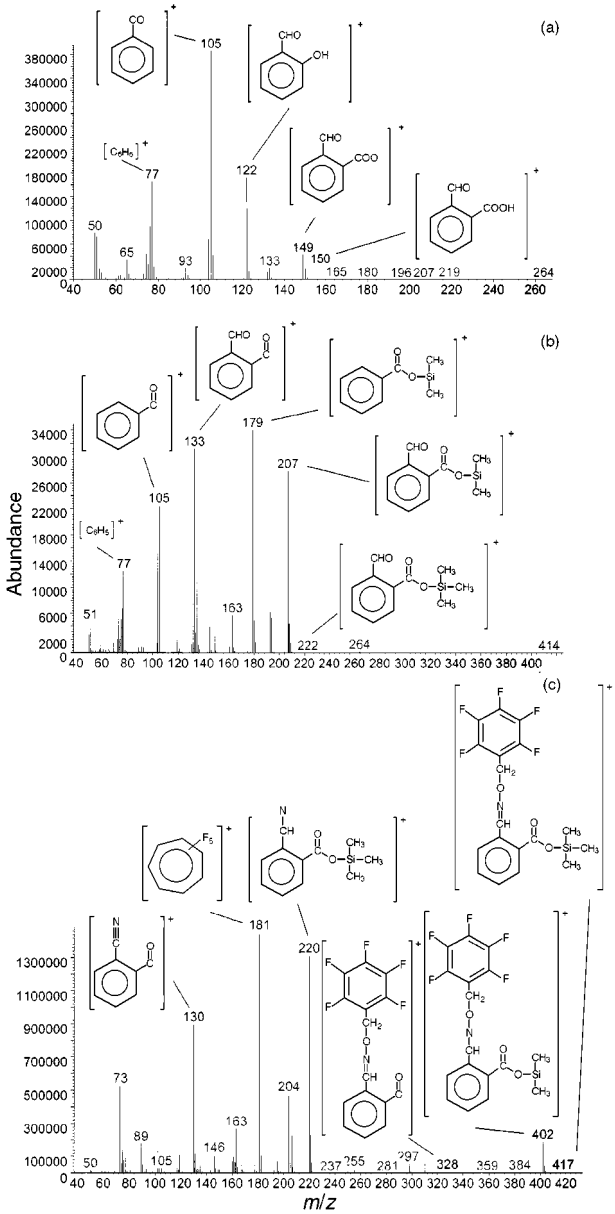

Fig 2 Mass spectra of 2-carboxybenzaldehyde (a) MSTFA derivative of 2-carboxybenzaldehyde (b) and PFBO-MSTFA derivative of 2-carboxybenzaldehyde (c).

Results and discussion

Identification and quantification of metabolites

Fluoranthene metabolic products were identified on the basis of molecular and fragment ions in the mass spectra and by comparison with the chromatographic retention time (*Rt*) of authentic compounds.

The quantification of metabolites was obtained using five point calibration curves determined by GC-MSD. Peak areas were demonstrated to be linear with the sample concentrations with correlation coefficients of 0.978 (9-fluorenone-1-carboxylic acid) and 0.991 (2-carboxybenzaldehyde) within the range of standards. Linear regression of standard deviation *versus* spiking level (five point calibration curve) was used to determine the standard deviation at zero concentration (*y* intercept, defined as SD_0). Instrument detection limits ($3 \times SD_0$), expressed in units of nanograms on-column (reached the detector) were 0.05 ng (2-carboxybenzaldehyde) and 0.9 ng (9-fluorenone-1-carboxylic acid). We determined the precision of the method by extracting a sample containing 9-fluorenone-1-carboxylic acid and 2-carboxybenzaldehyde six times. This resulted in a precision of 5.3% (2-carboxybenzaldehyde) and 10.9% (9-fluorenone-1-carboxylic acid).

GC-MS/SCAN

GC-MS/SCAN analyses of fluoranthene degradation samples demonstrated the presence of the metabolite benzoic acid as the TMS derivative in neutral and acidic extracts (0.967% and 0.695%). The metabolite 9-fluorenone-1-carboxylic acid was identified as the TMS ester (0.979%) and PFBO-TMS ester (2.090%) in the acidic extract. The presence of the metabolite 9-hydroxy-1-fluorene-carboxylic acid as the MSTFA derivative (0.994%) was also possible despite the absence of a molecular ion in the mass spectrum (*m/z* = 370). The chromatographic retention time and the remaining fragmentation pattern agree well with that of the authentic compound. Confirmation of the presence of this compound was expected by GC-MS/SIM analyses of the derivatised samples.

GC-MS/SIM

In the samples, no PFBO derivatives were detected by GC-MS/SIM. In the neutral and acidic extracts, the following metabolites were detected as TMS derivatives: 9-fluorenone-1-carboxylic acid (0.114% and 0.159%), 9-hydroxy-1-fluorene-carboxylic acid (0.008% and 0.032%), 2-carboxybenzaldehyde (0.107% and 0.237%) and benzoic acid (5.158% and 2.767%). The metabolite phenylacetic acid was identified in the acidic extract as the TMS ester (0.013%). The metabolite 9-fluor-

Table 3 Metabolite identification results after derivatisation with MSTFA and PFBHA.HCl by GC-MS/SCAN, SIM

	PFBO derivatives	TMS derivatives	PFBO-TMS derivatives
GC-MS/SCAN, *neutral extract*	u.d.l.[a]	Benzoic acid	u.d.l.
GC-MS/SCAN, *acidic extract*	u.d.l.	9-Fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, benzoic acid	9-Fluorenone-1-carboxylic acid
GC-MS/SIM, *neutral extract*	u.d.l.	9-Fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid	9-Fluorenone-1-carboxylic acid, 2-carboxybenzaldehyde
GC-MS/SIM, *acidic extract*	u.d.l.	9-Fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid, phenylacetic acid	9-Fluorenone-1-carboxylic acid, 2-carboxybenzaldehyde

[a] u.d.l. = under detection limit.

enone-1-carboxylic acid was also found as the PFBO-TMS ester (0.019% and 0.053%) in the neutral and acidic extracts. The metabolite 2-carboxybenzaldehyde was detected as a PFBO oxime or a PFBO-TMS ester in both extracts (0.006% and 0.006%). The identification of the derivatised form of this metabolite (PFBO oxime or PFBO-TMS ester) was not possible in the GC-MS/SIM mode because of the low concentration and the similarity in the structures of both derivatives, their fragmentation patterns and similar retention times. Since our main task was to identify fluoranthene metabolites and not their derivatised forms, this problem was not investigated further.

The efficiency of derivatisation is clearly shown from the results obtained (Tables 2 and 3). Table 2 shows that only two metabolites (9-fluorenone-1-carboxylic acid and 9-fluorenone) were identified without derivatisation, whereas after derivatisation (Table 3) five metabolites were identified: 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid.

A two step liquid–liquid extraction with ethyl acetate before and after sample acidification proved to be much more successful than a one step liquid–liquid extraction. The first extraction step removed most of the undegraded fluoranthene. The second is more convenient for extracting stable fluoranthene metabolites (pH 2.5).

As expected, our results confirmed that the GC-MS technique is more successful when operated in the SIM mode. In the neutral extract and SCAN mode, only benzoic acid was identified, while in the SIM mode 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid and 2-carboxybenzaldehyde were also identified. In the acidic extract, 9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluoren-carboxylic acid and benzoic acid were demonstrated to be present in the SCAN mode, while in the SIM mode besides these metabolites phenylacetic acid was also present. Even though the GC-MS/SIM method is known to be more sensitive, it can be applied only when known metabolites are predicted. The GC-MS/SCAN method, despite its lower sensitivity, has the advantage of complete metabolite fragmentation and therefore identification of unknown metabolites.

The literature reports that the most of the stable biodegradation metabolites are formed in low concentrations (< 1% of the initial substrate concentration).[17–20] In our study using bacterial strain *Pasteurella* sp. IFA, two metabolites [9-fluorenone-1-carboxylic acid (≅3%) and benzoic acid (≅8%)] were identified in higher quantities, while the concentration of the remaining metabolites was under 1%. Six metabolic products (9-fluorenone-1-carboxylic acid, 9-fluorenone, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid) were identified during fluoranthene biodegradation with *Pasteurella* sp. IFA, which accounted for 9% of the total stable metabolic products after 10 days of incubation. The same metabolites were identified during fluoranthene biodegradation studies by *Mycobacterium* sp. PYR-1.[7,11]

Conclusions

A combination of a two step liquid–liquid extraction procedure (neutral, acidic media) and derivatisation by silylation with MSTFA and oxime formation with PFBHA.HCl proved to be a powerful isolation technique for quantitative GC-MS analysis of stable fluoranthene biodegradation products. The method resulted in the identification of six metabolites: 9-fluorenone-1-carboxylic acid, 9-fluorenone, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phenylacetic acid. Derivatisation greatly improved the chromatographic and spectrometric properties of both authentic compounds and metabolites and this, combined with the low detection limits of GC-MS operated in the SIM mode, allowed the identification of previously unobserved metabolic products.

Ultimately, our research aims to investigate the potential of biodegradation as a means of remediating contaminated land. So far, from our study and from the literature,[7, 17–20] the level of degradation is below 10%, but given the high cost of complex equipment and the concerns regarding the environment, biodegradation remains a potential viable alternative, albeit on an industrial scale, for both *ex-situ* and *in-situ* remediation. Much remains to be done in terms of increasing the efficiency of degradation.

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