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Determination of alkylbenzene metabolites in groundwater by solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

Benzylsuccinate (BSA), methylbenzylsuccinate (methylBSA), and ethylbenzylsuccinate (ethylBSA) are unambiguous anaerobic biotransformation products from toluene, xylenes, and ethylbenzene decay, respectively, and may be used to indicate intrinsic bioremediation is occurring at hydrocarbon-contaminated sites. In order to improve upon current methods that detect and quantify anaerobic hydrocarbon metabolites in field samples, solid-phase extraction (SPE) and direct sample injection methods coupled with liquid chromatography–tandem mass spectrometry (LC–MS–MS) were evaluated. In laboratory studies, recoveries of authentic standards of non-deuterated or deuterated benzylsuccinates and toluates ranged from 80 to 106% with relative standard errors ranging from 2 to 4%. The method detection limits for these analytes using SPE–LC–MS–MS ranged from 0.006 to 0.029 μg/L whereas those for direct injection-LC–MS–MS ranged from 0.61 to 1.5 μg/L. Given the increased sensitivity of using SPE coupled with LC–MS–MS, this technique was then used to analyze for the presence of putative anaerobic alkylbenzene metabolites in groundwater from a hydrocarbon-contaminated site where single-well push–pull tests were conducted using deuterated aromatic hydrocarbons. Both deuterated and non-deuterated benzylsuccinates and toluates were successfully detected and quantified in field samples using this method.

Keywords: Water analysis; Environmental analysis; Alkylbenzenes; Benzylsuccinate; Benzenes; Toluene; Ethylbenzene; Xylenes

1. Introduction

The USA dependence upon groundwater is underscored by the fact that 99% of rural drinking water and 46% of total drinking water is obtained from aquifers [1]. Unfortunately, contamination of groundwater is a persistent, ubiquitous problem throughout the USA [2]. Aromatic hydrocarbons including benzene, toluene, ethylbenzene and the xylene isomers (BTEX) are introduced to the subsurface in the form of gasoline spills and leaks from underground storage facilities. The BTEX compounds are of particular interest due to their relative high water solubilities and toxicities [3,4] and because they are among the 33 synthetic organic contaminants most frequently found in drinking-water wells [5].

Limitations and costs associated with conventional remediation methods have generated interest in intrinsic bioremediation as an alternative to cleaning up contaminated groundwater [2,5,6]. Since available oxygen is quickly depleted in petroleum-impacted subsurface waters, anaerobic conditions often prevail [7], which limits intrinsic or enhanced bioremediation approaches to those relying on anaerobic metabolism.

The detection of metabolites that are unique to anaerobic alkylbenzene transformation are a definitive way to demonstrate that intrinsic or enhanced bioremediation is occurring at BTEX-contaminated sites [8,9]. Under anaerobic conditions, fumarate-addition reactions lead to the formation of unique metabolites including benzylsuccinate (BSA) from toluene, methylbenzylsuccinate (methylBSA) from xylenes, and ethylbenzylsuccinate (ethylBSA) from ethylbenzene, respectively [9–11]. While the transformation of these alkylbenzenes to their associated benzylsuccinate metabolites has been observed in numerous laboratory studies, as evidenced by numerous reviews [8,12–15], few reports describe the occurrence of BSA and methylBSA in groundwater at

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BTEX-contaminated sites [9,16–18]. To the best of our knowledge the detection of ethyl-BSA has not been reported for groundwater samples or groundwater tracer tests. Fewer yet are the number of reports that document benzylsuccinate formation during groundwater tracer tests [19,20]. Beller et al. [20] conducted a groundwater slug test in which toluene and xylenes were injected and the subsequent formation of BSA and methylBSA metabolites was observed. Reusser et al. [19] injected deuterated [$^2H_8$] toluene (toluene-d_8) and [$^2H_{10}$] *o*-xylene (*o*-xylene-d_{10}) and monitored the formation of deuterated [$^2H_8$] BSA (BSA-d_8) and [$^2H_{10}$] methylBSA (methylBSA-d_{10}) and reported first-order rates of formation. Rates obtained from in situ tracer tests are more likely to be representative of actual aquifer conditions than those obtained from microcosm studies [21].

Laboratory studies indicate that benzylsuccinates can be further transformed to toluate, phthalate, and benzoate [9,13]. While these metabolites are not unique to the anaerobic transformation of alkylbenzenes, the detection of their deuterated forms during groundwater tracer tests can be used to validate that alkylbenzenes are being metabolized in situ beyond the initial transformation to benzylsuccinates. To date, quantitative methods for the detection of the deuterated forms of toluate, phthalate, and benzoate have not been developed.

In support of previous laboratory and field studies, several analytical approaches were developed for the detection of BSA and methylBSA. Several studies have used liquid–liquid extraction coupled with gas chromatography–mass spectrometry (GC–MS) [20]; however, this method is time-consuming and uses relatively large volumes of solvent. Reusser and Field [18] coupled solid-phase extraction (SPE) with GC–MS analysis, which required extracting 1 L samples followed by derivatization with diazomethane, to achieve a method detection limit (MDL) of 0.2 μg/L. Subsequently, Beller [16] achieved an MDL of 0.3 μg/L for the analysis of BSA and methylBSA by direct injection liquid chromatography–tandem mass spectrometry (LC–MS–MS). This latter technique is more promising for rapid analysis because no extraction or derivatization procedures are required. However, additional analytical methodology is needed because [1] the concentrations of in situ benzylsuccinates can be at or below the MDLs of the current GC- and LC-based methods (e.g. <0.2–0.3 μg/L) and [2] methods are needed to detect metabolites resulting from the further decomposition of benzylsuccinates that may be produced during groundwater tracer tests.

Thus, the objective of this study was to develop and evaluate a SPE method coupled with LC–MS–MS for the determination of BSA, methylBSA, ethylBSA and their deuterated forms, and for toluates, deuterated toluates and deuterated benzoate. As part of this study, the SPE method was examined alongside a direct injection method similar to that outlined by Beller [8] in order to compare MDLs and to expand the methodology to include a greater suite of putative metabolites. To limit the scope of this project, the *m*-methylBSA isomer was selected; however, *o*- and *p*-methylBSA also could be determined by the methods described herein. Furthermore, because phthalates formed only at low concentrations in microcosm experiments [9], phthalates and their deuterated forms were not evaluated for this project. The developed and validated SPE method for the selected metabolites and their deuterated forms was then applied to the analysis of aromatic hydrocarbon-impacted groundwater samples.

2. Experimental

2.1. Reagents and standards

Standards of DL-benzylsuccinic acid (BSA, 99% purity) and benzoic acid (99.5%) were obtained from Alfa Aesar (Ward Hill, MA, USA). Standards of *m*-toluic acid (99%), *o*-toluic acid (99%), 2,4-difluorobenzoic acid (2,4-DFBA, 98%), and 2,4-dichlorobenzoic acid (2,4-DCBA, 98%) were obtained from Aldrich (Milwaukee, WI, USA). The surrogate standard, *p*-fluorobenzoic acid (4-FBA, 98%), was purchased from Sigma (St. Louis, MO, USA). The commercially-available deuterated standards, [$^2H_7$] *o*-toluic acid (*o*-toluic acid-d_7) (99.3%) and [$^2H_5$] benzoic acid (benzoic acid-d_5) (99.2%) were purchased from CDN Isotopes (Que., Canada). [$^2H_5$] Toluene (toluene-d_5), [$^2H_{10}$] *m*-xylene (*m*-xylene-d_{10}), and [$^2H_5$] ethylbenzene (ethylbenzene-d_5) for push–pull tests were also acquired from CDN isotopes. Acetone (HPLC grade) and methanol (Optima grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Hydrochloric acid was obtained from J.T. Baker (Phillipsburg, NJ, USA). Ethylbenzylsuccinic acid (ethylBSA) and *m*-methylbenzylsuccinic acid (*m*-methylBSA) were synthesized and purified according to published methods [22,23].

[$^2H_5$] Benzylsuccinate (BSA-d_5) and [$^2H_{10}$] *m*-methylbenzylsuccinate (*m*-methylBSA-d_{10}) were produced by enrichment cultures capable of biodegrading toluene or *m*-xylene under sulfate-reducing conditions [9]. Approximately 40 μmol (5 μL) of either d_5-toluene or *m*-xylene-d_{10} were amended to the enrichment cultures which were incubated until approximately half of the given alkylbenzene had been consumed. At this time, half of the culture supernatant was acidified, extracted with ethyl acetate, concentrated, silylated, and analyzed by GC–MS according to published methods [9] in order to confirm the presence of BSA-d_5 or *m*-methylBSA-d_{10}. Mass spectral profiles indicated that these deuterated metabolites were indeed formed (data not shown). The remaining culture supernatants were then subjected to SPE to concentrate metabolites. These concentrated metabolites were used as biologically-generated reference materials to confirm the presence of BSA-d_5 and *m*-methylBSA-d_{10} in groundwater samples analyzed by the LC–MS–MS method.

2.2. Groundwater samples

Groundwater samples were obtained from a BTEX-contaminated aquifer beneath a bulk fuel terminal near Portland, OR, USA. Previous investigations at this site had shown evidence for the in situ degradation of alkylbenzenes under anaerobic conditions [16,19]. Indeed, site groundwater was confirmed to be anoxic with measured dissolved oxygen levels $\leq$0.1 mg/L. In addition to background groundwater samples, groundwater samples containing nondeuterated and deuterated hydrocarbons and their putative anaerobic metabolites were obtained during single well push–pull experiments conducted at this site in a manner similar to those described by Reusser and Field [18]. Briefly, 250 L test solutions containing toluene-d_5, *m*-xylene-d_{10} and ethylbenzene-d_5 at concentrations ranging from 1210 to 1921 μg/L were injected into four hydrocarbon-contaminated wells, designated CR-12, CR-13, CR-14, and CR-20 [16,24]. In addition, the test solutions contained 100 mg/L bromide, which was used to normalize for dilution (e.g., a conservative tracer) [16,24]. Groundwater samples, collected from the wells over a 25-day period, were obtained using a Masterflex peristaltic pump (Barnant, Barrington, IL, USA) that was coupled to 0.6 mm nylon-braided poly(vinyl chloride) (PVC) tubing (Kuriyama, Santa Fe Springs, CA, USA) after first purging 3 well-casing volumes. Samples were collected in 1 L glass bottles preserved with 1.5% (v/v) 6 M HCl to achieve a pH of 2, and stored at 4 °C until analysis. An uncontaminated background well, CR-4, was also similarly sampled and used in method development experiments.

2.3. Solid-phase extraction (SPE)

Groundwater samples were brought to room temperature and vacuum filtered through a Whatman GDF 150, 1 μm glass microfibre filter (Whatman, Maidstone, UK). The filtered samples were spiked with 0.05 μg of the 4-FBA surrogate standard. An Empore SDB-XC 47 mm SPE disk (3M, Minneapolis, MN, USA) was placed in an MFS PP47, 47 mm polypropylene filter holder (Advantec/MFS, Dublin, CA, USA) that was fitted to a Supelco vacuum manifold (Sigma–Aldrich, Milwaukee, WI, USA). The disk was preconditioned by first wetting with 10 mL of acetone and allowing the disk to dry. The disk was further conditioned by passing 10 mL of isopropanol, 10 mL methanol and 5 mL of pH 2 reagent water sequentially without allowing the disk to go dry. A sample volume of 100 mL was then applied and the disk was allowed to dry for a minimum of 2 h under vacuum.

Compounds adhering to the SDB-XC disks were eluted by passing three 3 mL aliquots of methanol through the disk and collected in 15 mL vials (Supelco, Bellefonte, PA, USA). A 1 mL aliquot of reagent water was added to each vial along with 1 μg of 2,4-DCBA as an internal standard prior to reducing the volume to ~1 mL with heat (85 °C) under a steam of dry nitrogen using an N-Evap analytical evaporator (Organomation Associates, Berlin, MA, USA). During the concentration process, care was taken to ensure samples were not allowed to go dry, otherwise significant analyte losses were observed (data not shown). The sample vials were capped, cooled to room temperature, then the contents were transferred to a 2 mL autosampler vial.

2.4. SPE spike and recovery

Spike and recovery experiments were performed to determine the accuracy and precision of the SPE method. For these experiments, groundwater obtained from well CR-4 was used as the sample matrix since it was devoid of the analytes of interest. Five replicate 100 mL CR-4 groundwater samples were spiked to give a concentration of 1 μg/L of the following six analytes: [1] benzoate-d_5, [2] *m*-toluate, [3] *o*-toluate-d_7, [4] BSA, [5] ethylBSA and [6] *m*-methylBSA and 0.05 μg of the 4-FBA surrogate standard. All SPE extracts were spiked with 1 μg of the 2,4-DCBA internal standard prior to LC–MS–MS analysis. The concentrations of analytes spiked into CR-4 groundwater samples and concentrated by SPE were determined from calibration curves constructed from standards prepared in CR-4 groundwater.

2.5. SPE detection and quantitation limits

To determine the MDL for the SPE method, eight replicate 100 mL CR-4 groundwater samples were spiked to give 0.05 μg/L of each analyte and 0.5 μg/L of 4-FBA surrogate standard. An analyte concentration of 0.05 μg/L was selected because that concentration was estimated to be less than five times the predicted MDL. The MDLs for the SPE method were calculated as described by Glaser et al. [25]. Quantitation limits were defined as the concentrations that gave signal-to-noise values $\geq$10.

2.6. Direct injection

A set of spike and recover experiments was performed to determine the accuracy and precision of the direct injection LC–MS–MS method. To this end, seven replicate, 1 mL samples of CR-4 groundwater were spiked to contain 100 μg/L of each analyte and 1 μg of the 2,4-DCBA internal standard. Concentrations were quantified from calibration curves constructed from standards prepared in CR-4 groundwater. For purposes of comparison, the accuracy and precision of direct injection (25 μL injection volume) were determined. The MDL for direct injection was determined by spiking a set of eight replicate 1 mL samples of blank CR-4 groundwater to contain 5 μg/L of each analyte and 1 μg of 2,4-DCBA internal standard and analyzing the samples by direct injection.

2.7. Liquid chromatography–tandem mass spectrometry

All compounds were separated by a Waters 2690 Separations Module (Waters, Milford, MA, USA) liquid chromatograph fitted with a 150 mm × 2 mm Betasil C_{18} column (Thermo Electron Corp., Bellefonte, PA, USA). A mobile phase consisting of methanol–1 mM ammonium acetate buffer (pH 4) prepared in reagent water (43:57, v/v) was used in the isocratic mode with a 0.2 mL/min flow rate. A sample injection volume of 25 μL was used for all samples. The LC system was interfaced to a Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK). The mass spectrometer was operated in the negative electrospray ionization (ESI) mode with a desolvation temperature of 300 °C and a source block temperature of 80 °C. The desolvation and cone gas flows were set at 575 L/h and 92 L/h, respectively. For all compounds, the capillary voltage was set to 2.75 kV, the cone voltage was set to 24 V, and the multiplier voltage was set to 650 V. Argon (purity 99.998%) was used as the collision gas and the collision energy was set at a value between 12 eV and 14 eV, with the values optimized for each analyte. Collision cell pressure was set to be between 2.1×10^{-3} mbar and 2.2×10^{-3} mbar. A maximum of six transitions were monitored to achieve maximum sensitivity. For this reason, separate LC–MS–MS analyses were conducted to determine the deuterated and non-deuterated analyte concentrations.

Benzylsuccinic acid was spiked into ammonium salt buffers ranging from pH 3 to pH 7 and directly infused to compare signal strength for determination of the optimal buffer pH to be used in the mobile phase. A buffer pH of 4 gave the largest signal and was thus selected for all subsequent infusion experiments. To optimize tune settings and determine the appropriate transitions for quantitation, each analyte was prepared at a concentration ranging between 20 and 50 μg/L in a solution of methanol–10 mM ammonium acetate buffer in reagent water (pH 4) (50:50). The standard solutions were directly infused into the mass spectrometer at a rate of 10 μL/min via the syringe pump. Precursor ions of $[M - H]^-$ corresponded to the molecular ion minus an ionizable hydrogen atom. The product ions $[M - H–COO]^-$, corresponded to the loss of the CO_2 carboxyl moiety (*m*/*z* 44) and were used for quantitation [16].

2.8. Quantitation

Quantitation was performed by conventional internal standard calibration using calibration standards prepared both in reagent water and, where noted, in CR-4 groundwater. Calibration standards ranged from 5 to 500 μg/L for each analyte and contained 0.05 μg of the 4−FBA surrogate standard and 1 μg of the 2,4-DCBA internal standard. Absolute recoveries of analytes following SPE were determined from calibration curves constructed from analytes and the 2,4-DCBA internal standard while relative recoveries where determined from curves developed from analytes and the 4-FBA surrogate standard. Weighted linear regression ($1/x$) was used to generate calibration curves [26,27] from five calibration standards. Calibration curves were not forced through zero and R^2 values typically were 0.999.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Initial experiments were aimed at establishing the criteria for the detection and quantitation of deuterated analytes when authentic standards of deuterated analytes were not available. During the initial infusion experiments, precursor ions of $[M - H]^-$ with transition to product ions $[M - H–COO]^-$ were obtained for all the carboxylated analytes of interest and used for quantitation (Table 1). The transitions obtained for BSA and *m*-methylBSA were consistent with those reported by Beller [16]. Transitions of precursor ions to product ions were obtained for the non-deuterated analytes and their deuterated counterparts including benzoate/ benzoate-d_5, *o*-toluate/*o*-toluate-d_7, and for *m*-methylBSA/*m*-methylBSA-d_{10}. The consistent (e.g., predictable) difference in masses between the deuterated and

Table 1
Ion transitions used for identification and quantification for deuterated and non-deuterated analytes by LC–MS–MS

Analyte	Molecular mass	Precursor ion $[M - H]^-$ (*m*/*z*)	Product ion $[M - H–COO]^-$ (*m*/*z*)
Benzoate	122	121	77
Benzoate-d_5	127	126	82
o-, *m*-, *p*-Toluate	136	135	91
o-Toluate-d_7	143	142	98
Benzylsuccinate	208	207	163
Benzylsuccinate-d_5[a]	213	212	168
Ethylbenzylsuccinate	222	221	177
m-Methylbenzylsuccinate	222	221	177
m-Methylbenzylsuccinate-d_{10}[a]	232	231	187
2,4-Dichlorobenzoate (DCBA) (internal standard)	191	189	145
4-Fluorobenzoate (4-FBA) (surrogate standard)	140	139	95

[a] Biologically-generated (see Section 2).

nondeuterated analytes indicates that transitions can be reliably predicted for deuterated analytes from their nondeuterated counterparts when authentic standards are not available.

Under the chromatographic conditions used for this study, the deuterated analogs gave shorter (0.2–0.8 min) retention times than their non-deuterated counterparts (for example, compare *m*-toluate (Fig. 1a and b) and *m*-toluate-d_7 (Fig. 1c). Earlier elution of deuterated analytes is well documented and is due to the lower vibrational energy of the C–^{2}H bond compared to the C–H bond. The shorter, stronger C–^{2}H bonds are less polarizable than C–H bonds and exhibit less affinity for a non-polar stationary phase [28–31].

Response factors and/or retention times for equimolar concentrations of selected deuterated analytes were determined relative to their non-deuterated analytes. The deuterated/non-deuterated analyte pairs evaluated included benzoic/benzoic-d_5 and *o*-toluic/*o*-toluic acid-d_7. The BSA/ BSA-d_5 and *m*-methylBSA/ *m*-methylBSA-d_{10} pairs were not used to determine response factors because the biologically-generated BSA-d_5 and *m*-methylBSA-d_{10} concentrations in the microcosm were not known precisely. Response factors for deuterated analytes were within 10% of those obtained for non-deuterated analytes. In addition, the linear regressions of response factor versus analyte concentration for the deuterated analytes were ±10% of those for the nondeuterated analytes (data not shown). For these reasons, deuterated analytes for which authentic standards were not available (e.g., *m*-toluate-d_7) were quantified from calibration curves constructed from the corresponding authentic standards of non-deuterated analytes (e.g. *m*-toluate).

Attention was paid to the determination of benzoate-d_5 because it is a metabolite in common to ring-deuterated toluene and ethylbenzene. Although benzoate-d_4 would be expected from the transformation of ring-deuterated xylenes, sensitivity was maintained by keeping the number of multiple reaction monitored (MRM) ions to a minimum. For this reason, we chose to eliminate d_4-benzoate from our list of analytes. In addition, (nondeuterated) benzoate also was eliminated from further consideration because we were unable to obtain water samples that were blank with respect to benzoate for method development purposes.

3.2. Ion suppression

Signal suppression due to matrix effects have been reported for ESI mass spectrometry [32–35]. Signal suppression can occur because of competition between analyte and matrix-component ions for access to the droplet surface in the spray for emission as the gas-phase ion [33]. Since the goal of this research was to develop methods to detect and quantitate metabolites in groundwater samples, it was necessary to confirm that quantifiable analyses could be conducted with such matrices. Initially, 2,4-DFBA was selected for use as an internal standard for quantitation purposes for its structural similarity to the analytes under investigation and the low probability that it would occur in groundwater from

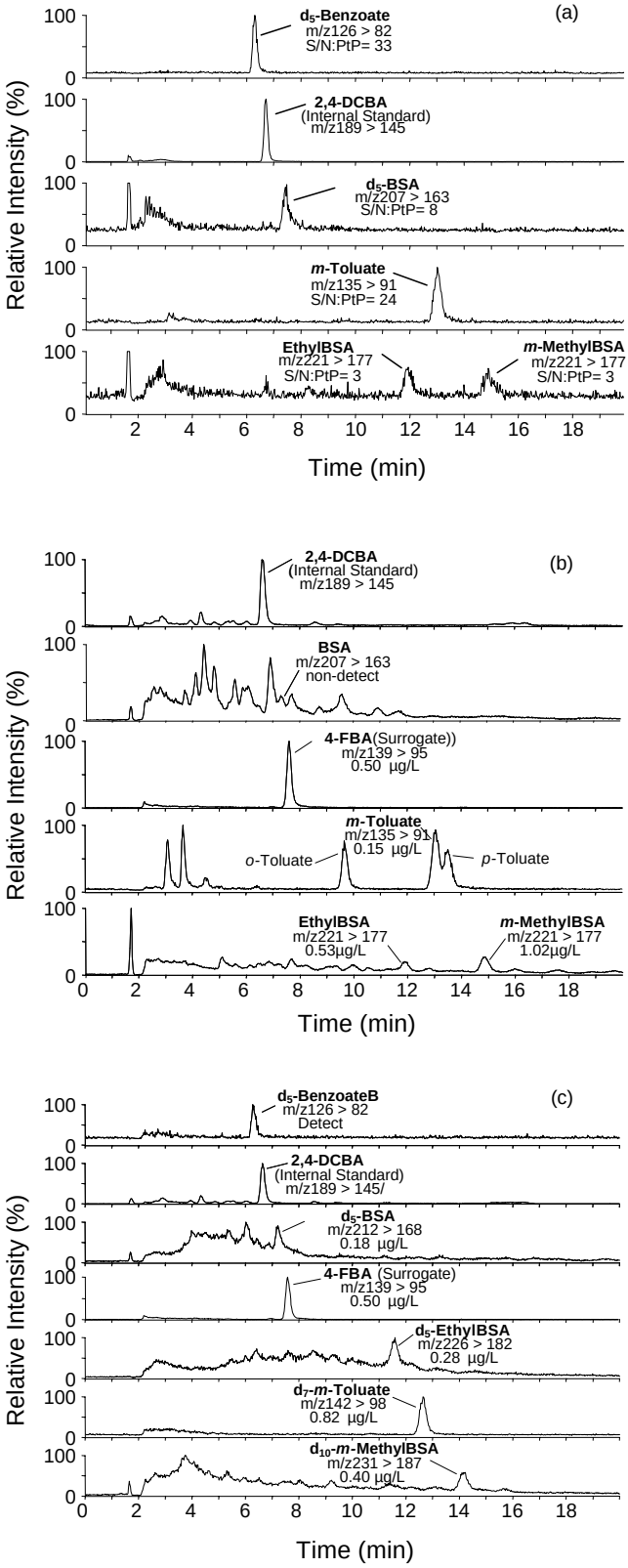

Fig. 1. Chromatograms for (a) a calibration standard with analytes (5 µg/L) spiked into CR-20 groundwater, (b) non-deuterated metabolites in CR-20 groundwater processed by solid-phase extraction, and (c) deuterated metabolites in CR-20 groundwater processed by solid-phase extraction. S/N: PtP = signal-to-noise measured peak-to-peak.

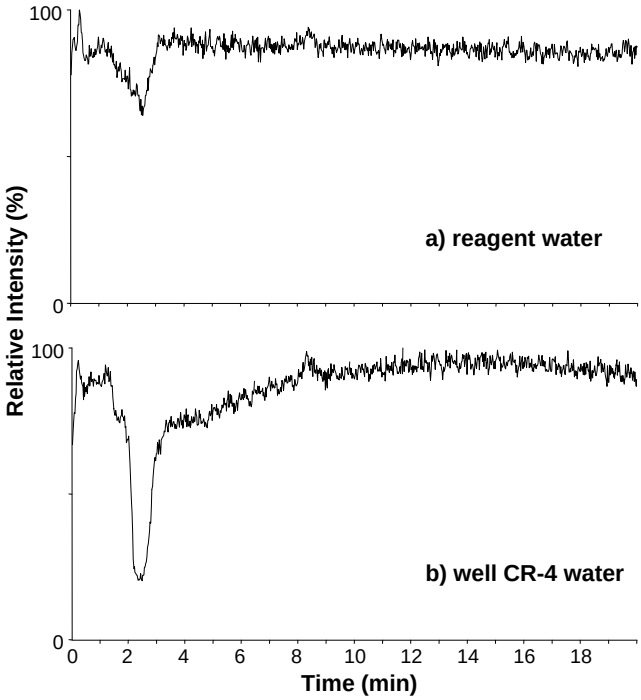

Fig. 2. Infusion chromatogram (20 min infusion of a mixture that was 100 μg/L of each analyte) of (a) a 25 μL injection of reagent water and (b) a 25 μL injection of blank CR-4 groundwater. Loss of signal between 2 and 8 min indicates ion suppression due to the groundwater matrix.

aromatic hydrocarbon-contaminated sites. However, further investigations revealed a significant loss of 2,4-DFBA signal when this standard was added to CR-4 groundwater. To test for ion suppression, the effluent line from the HPLC column was fit with a polyether ether ketone (PEEK) *t*-fitting and connected to a syringe pump that was used to continuously infuse (post-column) a 100 μg/L standard mixture of analytes into the mass spectrometer over a 20 min period in an approach described by Bonfliglio et al. [32]. Blank reagent water (Fig. 2a) and groundwater from well CR-4 (Fig. 2b) were then injected (25 μL each) separately into the HPLC system and the signal recorded over 20 min. Signal suppression, detected as a drop in the baseline beginning around 2 min, was greatest for the groundwater sample (Fig. 2a). It is likely that polar matrix components are responsible for the observed ion suppression because neither filtration nor solid-phase extraction lessened the level of ion suppression for 2,4-DFBA, which eluted at ~4.2 min. Alternatively, 2,4-DCBA, which eluted at ~6.5 min, was selected as the internal standard because its retention time was beyond the period of greatest ion suppression. Therefore, unless otherwise noted, 2,4-DCBA was used as the internal standard for all subsequent analyses.

3.3. Solid-phase extraction method

Empore extraction disks were selected for the solid-phase extraction procedure because of their ease of use and fast flow rates. The 47 mm SDB-XC disks were selected for this study because strong anion-exchange disks and C_{18} disks gave poor performance in earlier studies [18]. Initial breakthrough experiments indicated that up to 150 mL of sample could be passed through the 47 mL SDB-XC disk without analyte breakthrough including benzoate-d_5, *o*-toluate-d_7, benzylsuccinate, ethylbenzylsuccinate, and *m*-methylbenzylsuccinate (data not shown). A conservative volume of 100 mL was selected for subsequent use in method development.

The accuracy and precision of SPE was determined from spike and recovery experiments performed with analytes at high (1 μg/L) and low (0.05 μg/L) concentration in CR-4 groundwater. The absolute recoveries of analytes, determined relative to the internal standard, at a concentration of 1 μg/L ranged from 80 to 106% (Table 2). The precision of the method, as indicated by the relative standard error (RSE), ranged from 2 to 4% for the five replicate samples analyzed. The relative recovery of analytes, determined relative to the 4-FBA surrogate standard, ranged from 93 to 124% with RSE ranging from 1 to 4% (Table 2). From these data, 4-FBA was determined to be

Table 2
Precision and accuracy of the solid-phase extraction and direct injection methods for analytes spiked into blank CR-4 groundwater

Standard	Solid-phase extraction (1 μg/L)[a]			Direct injection (100 μg/L)[b]		
	Absolute recovery[c] (%)	R.S.E.[d] (%)	Relative recovery[e] (%)	R.S.E.[d] (%)	Absolute recovery[c] (%)	R.S.E.[d] (%)
Benzoate-d_5	80 ± 7	3	93 ± 5	2	101 ± 3	1
m-Toluate	96 ± 5	2	116 ± 6	2	99 ± 2	1
o-Toluate-d_7	84 ± 7	3	98 ± 5	2	103 ± 3	1
Benzylsuccinate	91 ± 9	4	106 ± 10	4	104 ± 11	4
Ethylbenzylsuccinate	106 ± 6	2	124 ± 2	1	101 ± 5	2
m-Methylbenzylsuccinate	101 ± 7	2	117 ± 4	1	102 ± 6	2
4-FBA (surrogate standard)	86 ± 5	2	NA	NA	103 ± 2	1

NA: not applicable.
[a] Five replicate samples of blank CR-4 groundwater spiked to contain 1.0 μg/L of each analyte; injection volume 25 μL.
[b] Seven replicate samples of blank CR-4 groundwater were spiked to contain 100 μg/L of each analyte; (direct) injection volume 25 μL.
[c] Absolute recovery relative to the internal standard; reported at the 95% confidence interval.
[d] R.S.E.: relative standard error = ((S.D./$\sqrt{n}$)/average recovery) × 100.
[e] Recovery relative to the surrogate standard; reported at the 95% confidence interval.

Table 3
Method detection limit (MDL) and quantitation limit (QL) for analytes by solid-phase extraction and direct injection into blank CR-4 groundwater

Analyte	Solid-phase extraction[a]		Direct injection[b]	
	MDL (μg/L)[c]	Quantitation limit[d] (μg/L)	MDL (μg/L)[c]	Quantitation limit[d] (μg/L)
Benzoate-d_5	0.007	0.05	0.79	5
m-Toluate	0.020	0.05	1.5	9
o-Toluate-d_7	0.016	0.05	0.73	4
Benzylsuccinate	0.022	0.15	1.5	4
Ethylbenzylsuccinate	0.020	0.15	1.4	10
m-Methylbenzylsuccinate	0.019	0.15	1.1	4

[a] Eight replicate samples of blank CR-4 groundwater were spiked to contain 0.05 μg/L of each analyte. Concentrations were determined from calibration curves constructed from standards prepared in blank CR-4 groundwater.
[b] Eight replicate samples of blank CR-4 groundwater were spiked to contain 5 μg/L of each analyte Concentrations were determined from calibration curves constructed from standards prepared in blank CR-4 groundwater.
[c] MDL: $t_{(n-1,1-\infty=0.99)} \times$ S.D. [25].
[d] Quantitation limit: concentration required to produce S/N = 10.

a suitable surrogate standard for these analytes in the SPE method.

The SPE method detection limit (MDL) was determined for blank CR-4 groundwater that was spiked with each analyte to a final concentration of 0.05 μg/L. The calculated MDL determined from these samples ranged from 0.007 to 0.022 μg/L (Table 3) and are a factor of 10 lower than that reported by others [16,18]. At a concentration of 0.05 μg/L, the chromatographic peaks for the analytes gave signal-to-noise ratios (S/N) that ranged from 3 to 4 for BSA, ethylBSA, and methylBSA and from 13 to 19 for benzoate-d_5, *m*-toluate, and *o*-toluate-d_7 (data not shown). The quantitation limits, defined as the concentration needed to produce a S/N $\geq$ 10, was defined as 0.05 μg/L for benzoate-d_5, *m*-toluate, and *o*-toluate-d_7 (Table 3). The estimated quantitation limit, determined as three times the 0.05 μg/L concentration that gave the observed S/N of $\geq$ 3, was 0.15 μg/L for BSA, ethylBSA, and *m*-methylBSA (Table 3).

3.4. Direct injection method

The recovery of analytes, when spiked into blank CR-4 groundwater at 100 μg/L, ranged from 99 to 104% with relative standard errors of 1–4% (Table 2). When analytes were spiked into blank CR-4 groundwater at a concentration of 5 μg/L, recovery ranged from 100 to 116% with relative standard errors of 1–4% (data not shown). The MDL for direct injection, determined from the replicate samples containing 5 μg/L of each analyte in blank CR-4 groundwater, ranged from 0.73 to 1.5 μg/L (Table 3). These MDLs are 2–5 times higher than those obtained in a previous direct injection study (0.3 μg/L) [15]. Thus, given that the SPE method described was found to be more sensitive than the direct injection method in our studies, we selected SPE coupled with LC–MS–MS to detect and quantify metabolites in hydrocarbon-contaminated groundwater.

3.5. SPE method demonstration on groundwater samples

Selected samples taken from single-well push–pull tests conducted at the Northwest Terminal site in which toluene-d_5, ethylbenzene-d_5, and *m*-xylene-d_{10} were injected into four wells. Samples from wells CR-12, CR-13, and CR-14 did not contain any of the target analytes above method detection limits (data not shown). This was surprising, since methylBSA was detected in these wells in previous examinations [16,18]. However, both non-deuterated (Fig. 1b) and deuterated (Fig. 1c) analytes were detected in samples collected from well CR-20 at various time points after injection. Of the non-deuterated benzylsuccinates, *m*-methylBSA was detected at the highest levels (0.20–1.14 μg/L) followed by ethylBSA (0.16–0.67 μg/L), while BSA was below the detection limit of 0.02 μg/L (Table 4; Fig. 1b). In well CR-20, *m*-toluate was detected (0.14–0.61 μg/L) as were *o*-toluate and *p*-toluate, although the latter were not quantified. The most abundant deuterated metabolite detected was *m*-toluate-d_7 (0.64–1.2 μg/L), followed by *m*-methylBSA-

Table 4
Concentrations (μg/L) of non-deuterated and deuterated metabolites of toluene, ethylbenzene, and xylenes and their deuterated analogs in groundwater obtained from well CR-20

Analyte	CR-20		
	Sample 6	Sample 16	Sample 17
Benzoate-d_5	0.64	<QL	<QL
m-Toluate	0.61	0.15	0.14
m-Toluate-d_7	1.2	0.82	0.64
Benzylsuccinate	ND	ND	ND
Benzylsuccinate-d_5	<QL	0.18	0.21
Ethylbenzylsuccinate	0.16	0.53	0.67
Ethylbenzylsuccinate-d_5	<QL	0.28	0.29
m-Methylbenzylsuccinate	0.20	1.02	1.14
m-Methylbenzylsuccinate-d_{10}	<QL	0.40	0.40

<QL: detected but at less than quantitation limit given in Table 3. ND: not detected above MDL of BSA (0.022 μg/L).

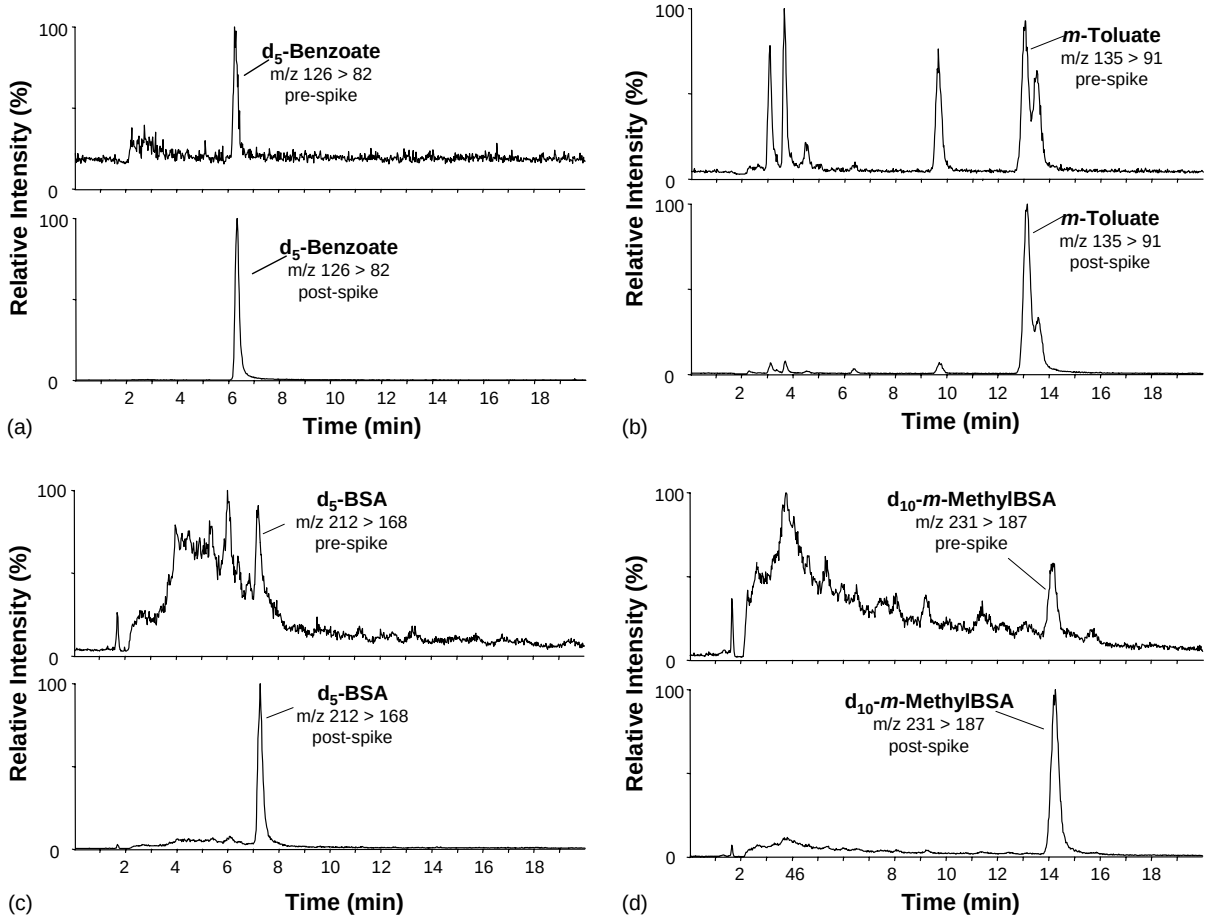

Fig. 3. Typical chromatograms for a single sample of CR-20 before and after spiking with deuterated and non-deuterated analytes including (a) benzoate-d_5, (b) *m*-toluate, (c) BSA-d_5, and d) *m*-methylBSA-d_{10}.

d_{10} (<QL–0.40 μg/L), ethylBSA-d_5 (<QL–0.29 μg/L), and BSA-d_5 (<QL–0.21 μg/L) (Table 4).

The identification of metabolites in CR-20 groundwater samples was verified by spiking the samples with standards. Authentic standards of benzoate-d_5 (Fig. 3a) and *m*-toluate (Fig. 3b) added to a CR-20 extract increased the appropriate peak area relative to that of the pre-spiked sample. The apparent recovery of benzoate-d_5 and *m*-toluate, spiked into extracts, ranged from 81 to 90% and from 90 to 113%, respectively (data not shown). The additions of BSA-d_5 (Fig. 3c) and *m*-methylBSA-d_{10} (Fig. 3d) increased the corresponding peak areas of these compounds in the CR-20 groundwater extracts relative to the pre-spiked sample, with recoveries ranging from 83 to 92% and from 110 to 156%, respectively (data not shown).

While Reusser and co-workers [18,19] were able to detect and quantify BSA in groundwater samples from the Northwest Terminal site, we were not able to detect its presence using SPE coupled with LC–MS–MS in our push–pull tests. Such a result may not be too uncommon, since other studies have mentioned that putative hydrocarbon metabolites are not always detected at every sampling event, even in the same wells [9,36]. However, we did detect and quantify *m*-methylBSA at concentrations lower than those possible using different methods followed in earlier studies [16,18].

In addition to our detection of benzylsuccinates as unique indicators of anaerobic alkylbenzene metabolism, we also detected toluates and benzoates. Although not considered unique to the anaerobic degradation of xylenes [9,13] since they may also be produced aerobically [20], these putative hydrocarbon metabolites were detected at higher concentrations than the signature benzylsuccinates at anoxic field sites [9]. The fact that we detected deuterated benzoate and *m*-toluate in addition to deuterated BSA and *m*-methylBSA in a push–pull test at an anoxic site does suggest that they were produced anaerobically and indicates further transformation of the benzylsuccinates thereby suggesting BSA and *m*-methylBSA are not dead-end products in this system. Detection of the unambiguous deuterium-labeled succinate metabolites coupled with the detection of similarly labeled biodegradation products of those succinate metabolites should be useful for obtaining unequivocal evidence for in situ alkylbenzene mineralization.

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