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Research Article

In Vitro Bioavailability of the Hydrocarbon Fractions of Dimethyl Sulfoxide Extracts of Petroleum Substances

Yu-Syuan Luo, Kyle C. Ferguson, Ivan Rusyn,[1] and Weihsueh A. Chiu [1]

Interdisciplinary Faculty of Toxicology and Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843

[1]To whom correspondence should be addressed. Fax: (979) 458-9866. E-mail: irusyn@cvm.tamu.edu and Fax: (979) 845-4106. E-mail: wchiu@cvm.tamu.edu.
Yu-Syuan Luo and Kyle C. Ferguson contributed equally to this study.

ABSTRACT

Determining the *in vitro* bioavailable concentration is a critical, yet unmet need to refine *in vitro*-to-*in vivo* extrapolation for unknown or variable composition, complex reaction product or biological material (UVCB) substances. UVCBs such as petroleum substances are commonly subjected to dimethyl sulfoxide (DMSO) extraction in order to retrieve the bioactive polycyclic aromatic compound (PAC) portion for *in vitro* testing. In addition to DMSO extraction, protein binding in cell culture media and dilution can all influence *in vitro* bioavailable concentrations of aliphatic and aromatic compounds in petroleum substances. However, these *in vitro* factors have not been fully characterized. In this study, we aimed to fill in these data gaps by characterizing the effects of these processes using both a defined mixture of analytical standards containing aliphatic and aromatic hydrocarbons, as well as 4 refined petroleum products as prototypical examples of UVCBs. Each substance was extracted with DMSO, and the protein binding in cell culture media was measured by using solid-phase microextraction. Semiquantitative analysis for aliphatic and aromatic compounds was achieved via gas chromatography-mass spectrometry. Our results showed that DMSO selectively extracted PACs from test substances, and that chemical profiles of PACs across molecular classes remained consistent after extraction. With respect to protein binding, chemical profiles were retained at a lower dilution (higher concentration), but a greater dilution factor (ie, lower concentration) resulted in higher protein binding in cell medium, which in turn altered the ultimate chemical profile of bioavailable PACs. Overall, this case study demonstrates that extraction procedures, protein binding in cell culture media, and dilution factors prior to *in vitro* testing can all contribute to determining the final bioavailable concentrations of bioactive constituents of UVCBs *in vitro*. Thus, *in vitro*-to-*in vivo* extrapolation for UVCBs may require greater attention to the concentration-dependent and compound-specific differences in recovery and bioavailability.

Key words: biotransformation; toxicokinetics; pharmacokinetics.

The need for substance-specific risk assessments of a large number of chemicals in commerce and the environment is increasing with stricter regulatory frameworks that are being adopted in many industrialized countries (Krimsky, 2017; Silbergeld *et al.*, 2015). Under the European Union's regulation for registration, evaluation, authorization and restriction of chemicals (REACH), detailed substance-specific risk assessments are required for high production volume substances or substances with mutagenic, carcinogenic, reproductive toxicity potential (European Chemicals Agency, 2012). Substances of unknown or variable composition, complex reaction products or biological materials (UVCBs), such as products of petroleum refining, are some of the most high production volume substances, yet they pose a number of unique challenges for evaluation under REACH (European Chemicals Agency, 2017). Petroleum substances have a very complex chemical profile; and the composition varies due to different refinery processes, intended applications, and sources of crude oils (Goyak *et al.*, 2016; Gray *et al.*, 2013).

A number of recent studies aimed to advance grouping and health hazard classification for UVCBs used petroleum substances as prototypical examples (Bierkens and Geerts, 2014;

Clark *et al.*, 2013; Dimitrov *et al.*, 2015; Grimm *et al.*, 2016, 2017; Kamelia *et al.*, 2017; Onel *et al.*, 2019). These case studies demonstrated how novel analytical and *in vitro* experimental data, now commonly referred to as "new approach methodologies" (NAMs), may be used to support regulatory decisions (Kavlock *et al.*, 2018). It is expected that the regulatory use of NAMs will increase in a variety of hazard and risk assessment applications, such as supporting read-across, prioritization and screening (European Chemicals Agency, 2016); however, concerns about the limitations of NAMs in decision making have been voiced (Berggren *et al.*, 2015; Gocht *et al.*, 2015). One of the concerns is that NAMs currently provide little insight into toxicokinetics and more data are needed to facilitate *in vitro*-to-*in vivo* extrapolation (IVIVE) of NAM data to human exposures scenarios (Bell *et al.*, 2018). Determining the *in vitro* bioavailable concentration is one critical element to IVIVE. It is needed not only to examine *in vitro* bioactivity, but also to establish relevant *in vivo* bioavailable concentrations, a task that is a challenge even for monoconstituent chemicals (Ferguson *et al.*, 2019; Sipes *et al.*, 2017; Wetmore, 2015), let alone mixtures or UVCBs.

For petroleum UVCBs, several factors complicate determination of the *in vitro* bioavailable concentrations. First, *in vitro* testing only assesses certain chemical classes of the molecules in a complex petroleum substance because samples must be extracted with solvents before testing (ASTM International, 2014; Carrillo *et al.*, 2019; McKee *et al.*, 2013). Among the numerous components in petroleum substances, polycyclic aromatic compounds (PACs; that include polycyclic aromatic hydrocarbons, heteroatoms, and alkylated molecules) are thought to be responsible for the developmental toxicity (Kamelia *et al.*, 2017; Tsitou *et al.*, 2015), endocrine disrupting potential (Lee *et al.*, 2017), and carcinogenicity (Goyak *et al.*, 2016; Varjani *et al.*, 2017). Concomitantly, petroleum products are usually subject to extraction with dimethyl sulfoxide (DMSO) using the IP 346 method (CONCAWE, 1994) to retain PACs in a solvent that is widely used as noncytotoxic vehicle for cell-based studies (Grimm *et al.*, 2016).

Second, because of the complex composition of DMSO extracts of petroleum substances, the nonspecific or protein binding of the individual components or whole classes of molecules may vary considerably thus affecting the bioavailable concentrations *in vitro*. Protein binding is an important component in IVIVE calculations (Bell *et al.*, 2018; Mielke *et al.*, 2017; Poulin *et al.*, 2016; Wetmore, 2015), yet no data on this property for complex UVCBs are available. Moreover, *in vitro* studies most often include dilution series to establish effective doses or points-of-departure that enable comparisons among substances and to substances known to be hazardous; however, it is not well established whether protein binding in the media and bioavailable concentrations scale proportionally with dilution of these complex substances.

To address a number of these challenges, we aimed in this study to characterize how 3 specific procedures used typicaly in *in vitro* testing protocols—DMSO extraction, addition of cell culture media, and serial dilution—impact chemical profiles and bioavailability of complex substances such as UVCBs. We performed this characterization both for defined mixtures of aromatic and aliphatic analytical standards as well as for refined petroleum products as case examples of actual UVCBs. This study thereby fills a critical knowledge gap in characterizing the *in vitro* bioavailable concentrations for complex substances, information which is needed to advance the use of NAMs in assessments of UVCBs.

MATERIALS AND METHODS

Overview of the experimental approach. This study aimed to characterize *in vitro* bioavailability of the hydrocarbon fractions of DMSO extracts of petroleum substances. We therefore designed 2 experimental arms (Figure 1). One was to investigate the effects of DMSO extraction using a neat mixture of monoconstituent hydrocarbon molecules containing 25 aromatic and 28 aliphatic hydrocarbons. The other was to use representative petroleum substance UVCBs from a diverse set of refining processes (heavy fuel oil [HFO], vacuum and hydrotreated gas oil [VHGO], straight run gas oil [SRGO], and other gas oil [OGO]).

In each case, the first aim was to assess the recovery from DMSO extraction. The products of DMSO extraction are used in cell-based *in vitro* experiments where they are further diluted with cell type-specific media that contains various amounts of proteins and other molecules that can sequester bioactive molecules in the extract and affect their bioavailability. Therefore, the second aim in each case was to assess degree of protein binding in cell culture media at a standard dilution of 200-fold (resulting in 0.5% DMSO). For refined petroleum substances, we additionally characterized bioavailability at a 20,000-fold dilution to reflect how concentration-response is typically assessed for *in vitro* tests.

Chemicals. Acetonitrile (Cat No.: A955-500), methanol (Cat No.: A456-500), DMSO (Cat No.: BP231-100), phosphate buffer saline (PBS, Cat No.: 20-012-027), and William's E Medium (no phenol red, Cat No.: NC0227405) were purchased from Fisher Scientific (Waltham, Massachusetts). iCell cardiomyocyte maintenance medium (Cat No.: M1004) was obtained from FujiFilm Cellular Dynamics (Madison, Wisconsin). Solid-phase microextraction (SPME) C18 fibers (Cat No.: 57234-U) and cyclohexane (Cat No.: 227048) were purchased from Sigma-Aldrich chemicals (St Louis, Missouri). Analytical standards of aromatic ($n = 25$) and aliphatic hydrocarbons ($n = 28$) were obtained from Absolute Standards (Hamden, Connecticut). Individual chemicals in the defined mixtures of the analytical standards are listed in Tables 1 and 2. Samples of petroleum substances from 4 separate refinement processes, SRGOs ($n = 5$), OGOs ($n = 2$), VHGOs ($n = 8$), and HFOs ($n = 3$) were provided by Concawe (Brussels, Belgium). One representative petroleum sample from each manufacturing stream (SRGO, CON-1; OGO, CON-07; VHGO, CON-15; and HFO, AB083/13) was used in this study.

DMSO extraction and recovery. We evaluated the recovery of polycyclic and aliphatic compounds, as well as their molecular classes, using the DMSO extraction procedure that was recently standardized for the application to the petroleum substances, as detailed elsewhere (ASTM International, 2014; Grimm *et al.*, 2016, 2017). In brief, 1 ml of the defined mixture or 4 g of each petroleum substance was mixed with 10 ml of cyclohexane and extracted twice with 10 ml of pre-equilibrated DMSO/cyclohexane (10:1) solution. A sample of each DMSO extract was analyzed by gas chromatography-mass spectrometry (GC-MS) (described below) and compared with analysis of original stock solutions to determine the efficiency of DMSO extraction for recovering aliphatic compounds, aromatic compounds, and molecular classes of hydrocarbons. For the experiment characterizing the effect of dilution on protein binding, an additional sample was prepared for each petroleum product where an additional 100-fold dilution with DMSO was performed;

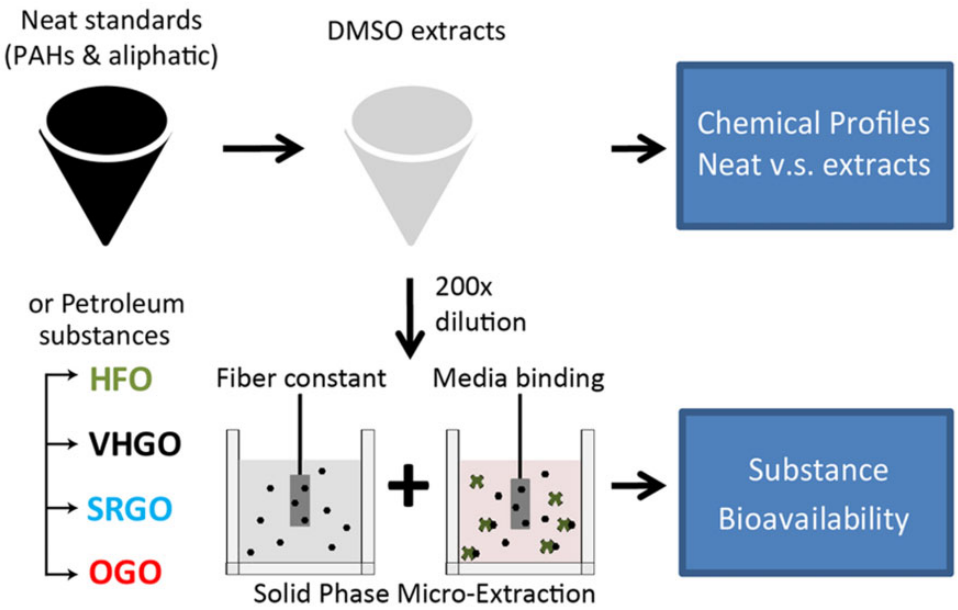

Figure 1. Schematic diagram for characterizing the effects of DMSO extraction on *in vitro* bioavailability. Quantitative results of aromatic and aliphatic compounds were obtained in neat defined mixture or petroleum refined products (heavy fuel oil, HFO; vacuum and hydrotreated gas oil, VHGO; straight run gas oil, SRGO; other gas oils, OGO), with or without DMSO extraction. *In vitro* bioavailability of tested substance was further determined by using solid-phase microextraction. Abbreviation: PAHs, polycyclic aromatic hydrocarbons.

Table 1. Aromatic Compounds Tested in this Study

Chemical Name	CAS Number	Molecular Weight	Log P
Acenaphthene	83-32-9	154.212	4.01
Acenaphthylene	208-96-8	152.196	4.00
Anthracene	120-12-7	178.234	4.53
Benzo(e)pyrene	192-97-2	252.316	6.19
Benzo[a]anthracene	56-55-3	228.294	5.76
Benzo[a]pyrene	50-32-8	252.316	6.13
Benzo[b]fluoranthene	205-99-2	252.316	6.09
Benzo[g, h, i]perylene	191-24-2	276.338	6.74
Benzo[k]fluoranthene	207-08-9	252.316	6.12
Biphenyl	92-52-4	154.212	4.07
Chrysene	218-01-9	228.294	5.77
Dibenzo[a, h]anthracene	53-70-3	278.354	6.78
Dibenzothiophene	132-65-0	184.26	4.42
Fluoranthene	206-44-0	202.256	5.18
Fluorene	86-73-7	166.223	4.15
Indeno[1,2,3-cd]pyrene	193-39-5	276.338	6.77
Napthalene	91-20-3	128.174	3.32
1-Methylnaphthalene	90-12-0	142.201	3.96
2-Methylnaphthalene	91-57-6	142.201	3.94
2,6-Dimethylnaphthalene	581-42-0	156.228	4.35
1,6,7-Trimethylnaphthalene	2245-38-7	170.255	4.65
Perylene	198-55-0	252.316	6.19
Phenanthrene	85-01-8	178.234	4.52
1-Methylphenanthrene	832-69-9	192.261	5.06
Pyrene	129-00-0	202.256	5.05

Table 2. Aliphatic Compounds Tested in This Study

Chemical Name	CAS Number	Molecular Weight	Log P
n-C10	124-18-5	142.286	5.7
n-C11	1120-21-4	156.313	6.1
n-C12	112-40-3	170.34	6.5
n-C13	629-50-5	184.367	6.9
n-C14	629-59-4	198.394	7.2
n-C15	629-62-9	212.421	8.0
n-C16	544-76-3	226.448	8.5
n-C17	629-78-7	240.475	8.9
Pristane	1921-70-6	268.529	9.0
n-C18	593-45-3	254.502	9.4
Phytane	638-36-8	282.556	9.3
n-C19	629-92-5	268.529	9.7
n-C20	112-95-8	282.556	10.1
n-C21	629-94-7	296.583	10.4
n-C22	629-97-0	310.61	10.1
n-C23	638-67-5	324.637	10.2
n-C24	646-31-1	338.664	10.6
n-C25	629-99-2	352.691	10.9
n-C26	630-01-3	366.718	11.2
n-C27	593-49-7	380.745	11.5
n-C28	630-02-4	394.772	11.8
n-C29	630-03-5	408.799	12.1
n-C30	638-68-6	422.826	12.2
n-C31	630-04-6	436.853	12.5
n-C32	544-85-4	450.88	13.0
n-C33	630-05-7	464.907	13.3
n-C34	14167-59-0	478.934	13.7
n-C35	630-07-9	492.961	14.0

yielding second working concentration that is 1% of the original DMSO extract.

SPME experiment for protein binding analyses. Protein binding analysis follows previously described methods with some modifications (Musteata *et al.*, 2006; Peltenburg *et al.*, 2015). Briefly, the C_{18} SPME fibers were preconditioned in methanol/Milli-Q water solution (50%:50%, v/v) for 30 min. iCell cardiomyocyte maintenance media (FujiFilm Cellular Dynamics) was thawed to room temperature and penicillin-streptomycin was added according to manufacture instructions. DMSO extracts from the defined

mixture or petroleum substances were further subjected to 200-fold dilution with iCell cardiomyocyte media, yielding the final DMSO content of 0.5%. Once prepared, sample (100 µl) was transferred into a 200 µl glass insert with a 2 ml amber glass vial to equilibrate on an orbital shaker (500 rpm) at 37°C for 1 h. SPME fibers were then inserted through the vial cap septa and placed in the incubator on an orbital shaker (500 rpm) for 3 h. After the 3-h incubation, SPME fibers were removed, rinsed briefly with Milli-Q water, and then placed in 100 µl of acetonitrile on an orbital shaker (500 rpm) at room temperature for 30 min. Standard solutions were prepared in PBS, following the same procedures as described earlier. All experiments were carried out in triplicates.

Calculation of SPME protein binding. The calculation of bioavailable chemical fractions using SPME followed procedures described elsewhere (Ferguson *et al.*, 2019). Briefly, the partition behavior between unbound chemical and SPME fiber was defined as the fiber constant (f_c) by analyzing standard solutions of chemical in PBS buffer according to the equation 1, where $C_{0,s}$ is the initial concentration before fiber extraction and $C_{e,s}$ represents the extracted concentration from SPME fiber for the PBS buffer control group.

$$f_c = \frac{C_{e,s}}{C_{0,s} - C_{e,s}} \quad (1)$$

Next, the bioavailable chemical fraction (C_{free}) was determined in the previously prepared sample containing cell culture medium (equation 2), where C_e represents the extracted concentration from SPME fiber for the experimental group.

$$C_{free} = \frac{C_e}{f_c} \quad (2)$$

The final total concentration (C_t) of chemical, including the bound and unbound fractions in the sample, was determined using the equation 3, where C_0 represents the initial chemical concentration prior to fiber extraction in the experimental group.

$$C_t = C_0 - C_e \quad (3)$$

Ultimately, the percentage unbound (% bioavailable) is calculated from the total and bioavailable concentration of the chemical (equation 4).

$$\%\ \text{Unbound} = \left(1 - \frac{C_t - C_{free}}{C_t}\right) \times 100 \quad (4)$$

Sample analysis by selective ion monitoring GC-MS. Analytical method was designed in accordance with ASTM D5739 with some modification (ASTM International, 2000). Analysis was performed by an Agilent 6890 N gas chromatogram with an Agilent 5975C mass spectrometer (Santa Clara, California) operating in electron impact ionization mode. Data were collected via selective ion monitoring mode, for additional instrument parameters see Supplementary Table 1. Sample (1 µl) was injected with splitless mode to an Agilent DB-5ms column (Agilent DB-5 30.0 × 250 mm, 0.25-µm film thickness). Chromatographic separation was achieved using the following oven gradient: (1) initial injection port temperature set to 250°C with initial oven temperature set to 55°C; (2) Oven temperature increased 6°C/min to 270°C; (3) Oven temperature increased 3°C/min to 300°C; (4) Final oven temperature of 300°C held for 17 min. Total run time was 65 min.

DMSO extracts of petroleum substances and SPME samples were diluted with 4% sodium chloride solution (1:2) and extracted twice with 2 ml and 1 ml of pentane subsequently. Excess water and DMSO were removed through the addition of sodium sulfate. Pentane extract was transferred to a separate vial and remaining sodium sulfate was rinsed 3 times with 1 ml of pentane. Total pentane extracts were then transferred to 25-ml glass concentrator tubes, submerged into a hot water bath, and concentrated to approximately 100 µl prior to GC-MS analysis. Semiquantitative analysis was performed through the integration of peak response area for each analyzed ion relative to the summation of peak areas across the entire sample. Analyzed ions are categorized according to carbon number and molecular class of the parent molecule to generate a 2D matrix, evaluating the percent composition of the individual compound over the total sample. Subsequent evaluation pertaining to a specific molecular class or carbon number is obtained through summation of the entire column or row within the matrix, for 2D matrix example see Supplementary Table 2.

Neat petroleum substances were analyzed following a similar protocol as described earlier, with the exception of performing a 1:500 spilt injection and conducting a full scan of ion mass ranging from 55 to 300 m/z. The instrumental parameters are shown in Supplementary Table 1. Neat petroleum samples were analyzed without any solvent preparation prior to GC/MS split injection.

RESULTS

Recovery from DMSO Extraction of a Defined Mixture

The recoveries of various hydrocarbon chemical species from DMSO extractions of defined mixtures of the analytical standards are shown in Figure 2. It is well established that DMSO efficiently extracts polycyclic, but not aliphatic compounds (Natusch and Tomkins, 1978). We found efficient, but variable, recovery rates for the polycyclic molecules and very low for the aliphatic compounds. Aliphatic hydrocarbons showed low recovery (<2.5%) during the DMSO extraction process. When grouped by a molecular class, the average recovery from DMSO extraction was 1.4% ± 1.1% for n-paraffins and 1.1% ± 1.0% for iso-paraffins. Recovery of various polycyclic molecules ranged from 55.7% to 95.3%. When grouped by a molecular class, the average recovery from DMSO extraction was 77.3% ± 5.0% for diaromatic, 72.7% ± 4.7% for naphthenic diaromatic, 86.2% ± 4.7% for triaromatic, 89.4% ± 3.7% for aromatic sulfur bearing, and 78.5% ± 8.5% for polyaromatic compounds.

In Vitro *Bioavailability in Cell Culture Media of DMSO Extracts of a Defined Mixture*

The bioavailable fractions of polycyclic hydrocarbon compounds determined by SPME are shown in Figure 3. Aliphatic standards were not assessed due to their low recovery during DMSO extraction. The individual molecular classes of polycyclic hydrocarbons showed similar partitioning between the stock solution and the bioavailable amount (Figure 3A). This observation confirmed previous reports (King *et al.*, 2003) that SPME is a high-fidelity technique for polycyclic hydrocarbon extraction because it closely resembles the composition of a DMSO extract. The corresponding bioavailable fractions of polycyclic hydrocarbon compounds and their molecular classes are shown in

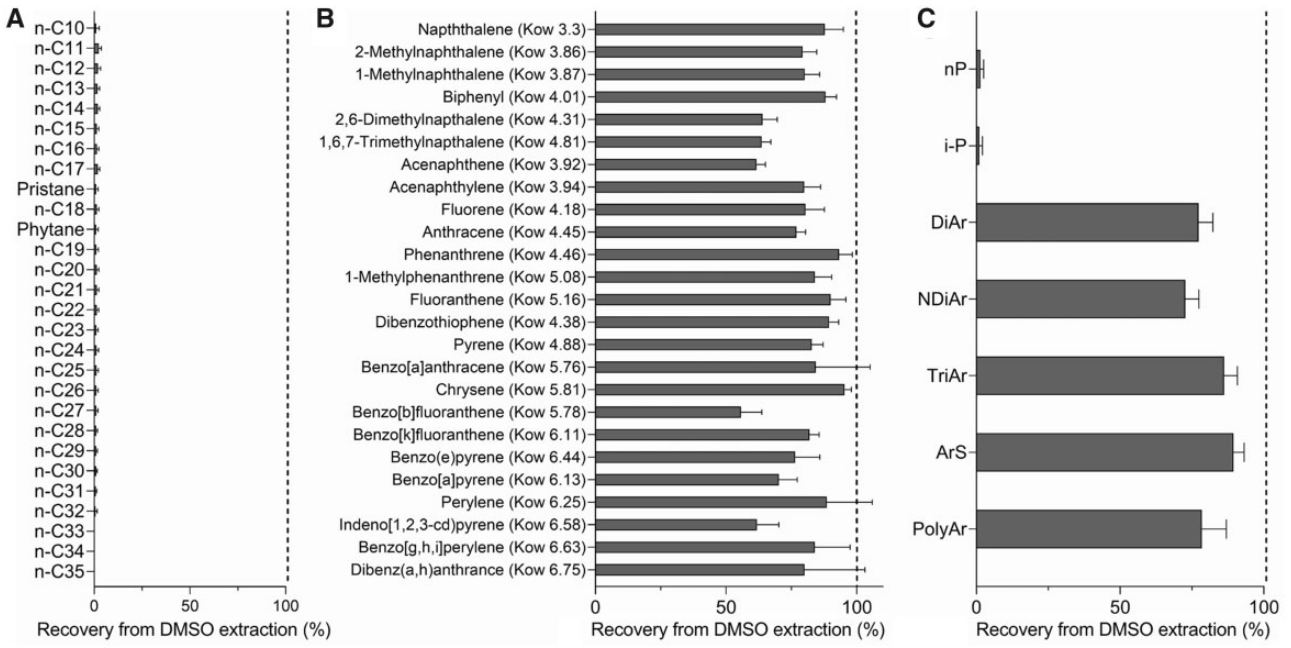

Figure 2. Efficiency of the DMSO extraction procedure (ASTM International, 2014) for the recovery of the (A) aliphatic compounds, (B) aromatic compounds, and (C) molecular classes of hydrocarbons from a defined mixture of analytical standards. Abbreviations: n-paraffin, n-P; iso-paraffin, i-P; diaromatic, DiAr; naphthenic diaromatic, NDiAr; triaromatic, TriAr; aromatic sulfur bearing, ArS; polyaromatic, PolyAr. Mean and SD of the recovery (in %) from DMSO extractions ($n = 3$) are shown.

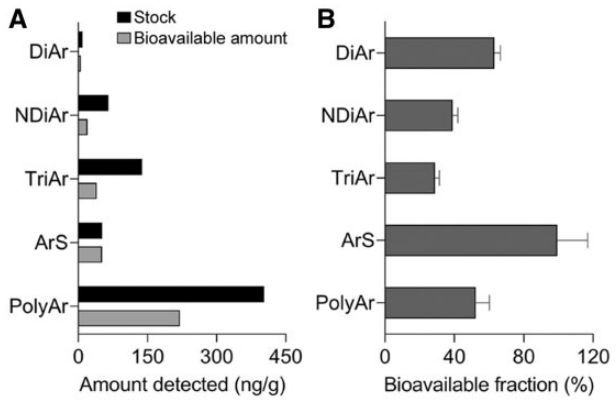

Figure 3. Determination of the bioavailable fraction of the polycyclic hydrocarbon compounds using solid-phase microextraction (SPME) in a defined mixture of analytical standards. A, Effects of SPME measurements on the percent distribution of the polycyclic hydrocarbon compounds. Black bars represent the total amount of the polycyclic hydrocarbon in the neat defined mixture. Gray bars indicate the bioavailable amount of the polycyclic hydrocarbon determined via SPME. B, Overall bioavailable fractions of different polycyclic hydrocarbon molecular subclasses ($n = 3$, mean ± SD).

Figure 3B. The bioavailable fraction was the highest for aromatic sulfur-bearing compounds (99% ± 18%), followed by diaromatic compounds (63% ± 3.5%), polyaromatic compounds (52% ± 7.9%), naphthenic diaromatic compounds (39% ± 3.0%), and triaromatic compounds (29% ± 3.1%).

Recovery from DMSO Extraction of Refined Petroleum Products

The chemical profiles and recoveries of the aliphatic and polycyclic hydrocarbon compounds in 4 refined petroleum products (SRGO, OGO, VHGO, and HFO) before and after the extraction with DMSO are compared in Figure 4. DMSO extraction efficiently recovered polycyclic hydrocarbons across all 4 refined petroleum products. Aliphatic compounds (ie, n-paraffin, n-P and iso-paraffin, i-P) accounted for 60.8%–77.0% of the total composition of the neat SRGO, OGO, and VHGO, but only <0.2% in their corresponding DMSO extracts (Figure 4A and B). When only polycyclic hydrocarbon compounds are considered, we found that the proportions of different molecular classes were largely similar between the neat petroleum refining substances (Figure 4C) and their corresponding DMSO extracts (Figure 4D). However, based on the mass, DMSO extracts contained 38%–59% of DiAR, 32%–87% of NDiAR, 38%–85% of TriAR, 8.2%–77% of ArS, and 19%–67% of PolyAR as compared with their neat form. These results show that despite the partial loss of polycyclic hydrocarbon compounds during extraction, the overall compositional profile of the molecular classes is preserved.

In Vitro *Bioavailability in Cell Culture Media of DMSO Extracts of Refined Petroleum Products*

The bioavailable fractions of polycyclic hydrocarbon compounds determined by SPME are shown in Figure 5. We found that the lower dilution factor (ie, higher concentration of the tested substance) yielded higher *in vitro* bioavailable fractions of the polycyclic hydrocarbon compounds; however, large differences in the bioavailability of different molecular classes were observed at higher dilutions (Figure 5A and B). Considering the efficiency of recovery from DMSO and the bioavailable fraction for each molecular class of polycyclic hydrocarbon compounds, we found that the bioavailable profile of these molecules in experimental wells with different dilutions of the original DMSO extract differ to a large extent (Figure 5C and D). For the 200-fold diluted samples with higher *in vitro* bioavailable fractions, the ultimate chemical profiles of the polycyclic hydrocarbon compounds *in vitro* were largely similar to those obtained with the DMSO extracts. However, for the 20,000-fold diluted samples with lower *in vitro* bioavailable fractions, ArS and PolyAr compounds were largely absent from the media for the samples of OGO and VHGO substances. Overall, we conclude that different

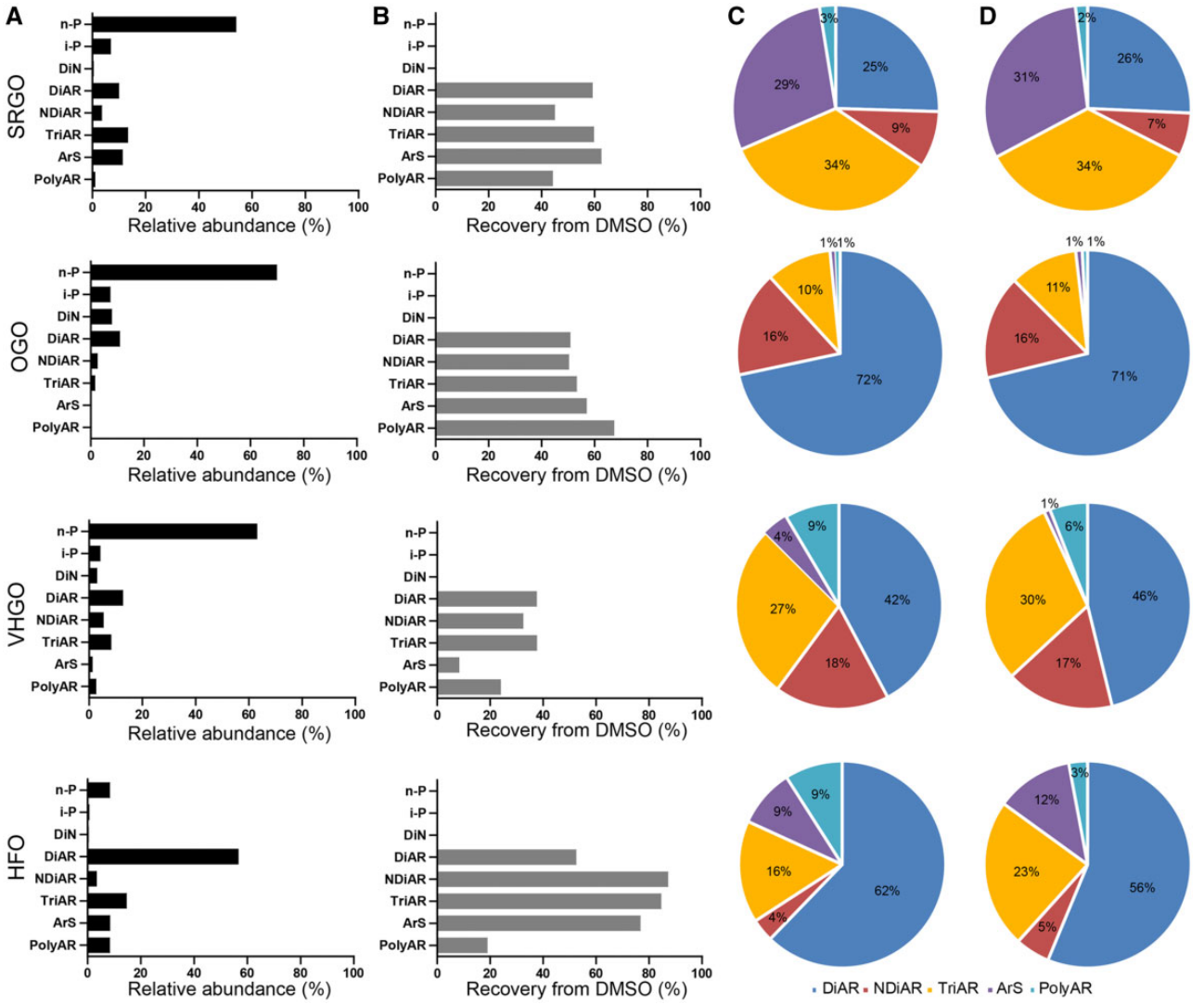

Figure 4. Chemical profiles of the aliphatic and polycyclic hydrocarbon compounds in neat petroleum substances and their DMSO extracts. A, Relative abundance of the molecular classes of hydrocarbon molecules in the neat substance of each type. B, Recovery of the same molecular classes after DMSO extraction. Chemical profiles of the polycyclic hydrocarbon compounds in the neat petroleum products (C) and their corresponding DMSO extracts (D). Pie chart slices in (C) and (D) correspond to the following molecular classes of the polycyclic hydrocarbon compounds: DiAR, dark blue; NDiAR, red; TriAR, yellow; ArS, purple; PolyAR, light blue.

dilution factors would result in distinct chemical profiles of the polycyclic hydrocarbon compounds *in vitro*.

DISCUSSION

A continuing challenge in the use of *in vitro*, cell-based NAMs for risk assessment is the confident extrapolation of test concentrations to the *in vivo* setting. With the development of *in vitro* ADME assays, pharmaceutical industries and regulatory institutions utilize the *in vitro* kinetic parameters to estimate in *vivo* bioavailability and conduct IVIVE (Bohnert and Gan, 2013; Camenisch and Umehara, 2012; Volpe, 2016; Waters *et al.*, 2008; Wetmore *et al.*, 2012). However, when it comes to UVCBs, IVIVE becomes even more complicated because of the nature of the complex composition, the additional extraction procedures, and other *in vitro* factors that may influence the *in vitro* bioavailable concentrations. In this study, for the first time, we systematically evaluated the effects of DMSO extraction, protein binding in cell culture media, and dilution factors on *in vitro* bioavailable concentrations of aliphatic and aromatic compounds in both a defined mixture of analytical standards and in refined petroleum substances.

We found that DMSO extraction procedures selectively retrieve the PACs from the tested substances. These observations are concordant with those reported in the literature (Natusch and Tomkins, 1978; Wang *et al.*, 2000). However, in general, the recoveries of aromatic compounds from DMSO extractions were higher in the defined mixture of analytical standards as compared with those in petroleum substances. The interactions between other substances in petroleum products and aromatic compounds could result in the lower overall recovery of aromatic compounds for petroleum substances. Interestingly, ArS and PolyAr reported lower recoveries from DMSO extractions compared with other PACs molecular classes in petroleum products, except in OGO. Lipophilicity of a compound could be an important factor to determine one's recovery from DMSO extraction, as DMSO preferably extracts compounds with lower lipophilicity. A higher lipophilicity of ArS (logP = 4.38), PolyAr (logP = 4.88–6.75), as to

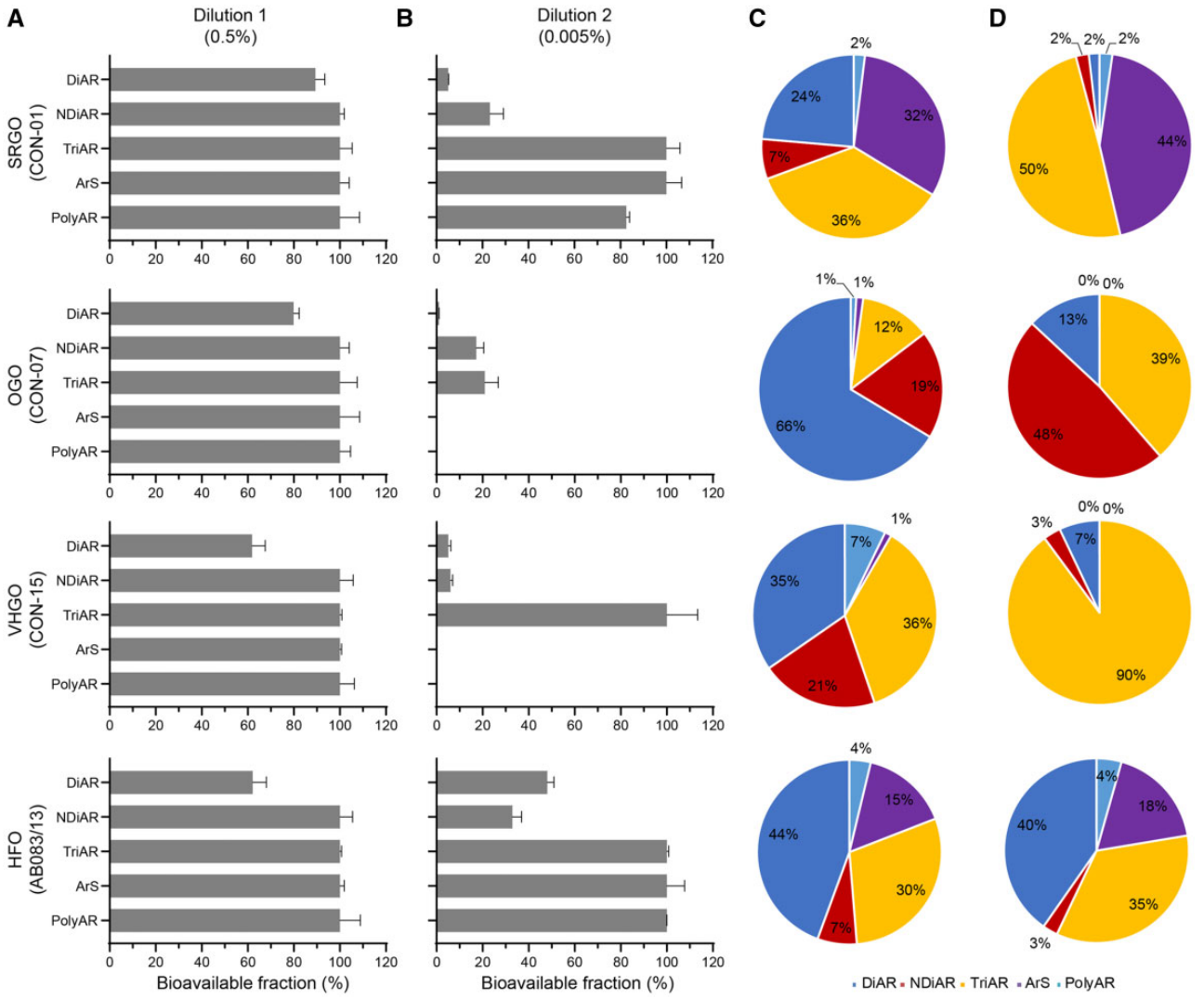

Figure 5. Effects of the dilution factors on the bioavailability of various polycyclic hydrocarbon molecular classes in DMSO extracts of petroleum substances. A and B, are bioavailable fractions at different dilutions as compared with the neat DMSO extract of each petroleum substance. Gray bars represent the bioavailable fractions of aromatic compounds in cell medium (cardiomyocyte), which were derived from the protein binding analysis via SPME at 2 dilutions, 0.5% (A) and 0.005% (B) of the beginning DMSO extract. (C) and (D) are chemical profiles of the polycyclic hydrocarbon molecular classes in the cell culture media at different dilution. (C) is for 0.5% and (D) is for 0.005% final concentration of the neat DMSO extract.

aliphatic compounds (logP > 5.65, decane) may explain the lower recovery of these species in DMSO extracts.

Despite the loss in the absolute amount of the aromatic compounds, the relative abundances of PACs remained intact after the DMSO extraction, both for the defined mixture of the analytical standards and for refined petroleum substances. Given that the aromatic compounds are thought to be the "bioactive" components of the petroleum substances, our results demonstrate that the use of DMSO extracts for *in vitro* testing may appropriately reflect the *in vitro* bioactivity of petroleum substances.

However, the DMSO extracts usually are tested *in vitro* in different dilutions. Concentration-dependent (Giacomini and Blaschke, 1984; Schleibinger *et al.*, 2015; Stoeckel *et al.*, 1981), or concentration-independent (Moschitto and Greenblatt, 1983; Taneja *et al.*, 2015) plasma protein binding has been reported in drugs. We therefore compared protein binding of UVCBs at different dilution factors. We found that the concentration of the DMSO extracts of tested substances played a critical role in determining *in vitro* bioavailable concentrations, where lower protein binding was found at higher *in vitro* concentrations (ie, a smaller dilution factor). This result is not surprising because protein levels in media are likely the limiting factor for the protein binding of UVCBs. If the binding sites of the proteins are fully occupied at higher *in vitro* concentrations, additional compounds will be free in cell culture medium, which in turn results in a higher bioavailable concentration. Traditionally, protein binding parameters are obtained individually for each chemical, usually at concentrations (1 or 10 μM) that are unlikely to saturate the proteins in test solutions (Rotroff *et al.*, 2010). Mixtures and UVCBs are likely to saturate protein binding more easily than the individual chemicals because every component in a UVCB could contribute to the protein binding *in vitro*. Our results demonstrate that the investigation of concentration-dependent protein binding is crucial to determine *in vitro* bioavailable concentrations, especially for UVCBs. The dilution of the DMSO extract of petroleum substances not only reduced the absolute amount of each chemical constituent, but also changed the final

chemical profile of polycyclic aromatic hydrocarbon compounds in the cell culture media. The assumed linear relationship between tested concentration and bioactivity may be challenged due to the alteration in chemical profiles. These results may have implications beyond UVCBs to mixtures more generally, as people are not exposed to individual chemicals one at a time in the environment. However, human plasma has much higher protein content than cell culture media, so the impact of the potential saturation may be ameliorated.

The protein binding efficiency in cell culture media of PACs reported in this study is far lower than that reported previously for human plasma (Williams *et al.*, 2017). For example, the unbound fraction of fluorene (18.86%, logP = 4.18) was 9.43-fold higher than that reported in the U.S. EPA CompTox Chemistry Dashboard (Williams *et al.*, 2017). There are 3 factors that can introduce this discrepancy in protein binding values. First, the protein binding values were obtained in different sample compositions. Even though the cell culture medium used in this study was fortified with a serum-containing supplement, the protein content was still lower than that in human plasma. Second, the values obtained in this study were under mixture conditions; however, those reported in the Chemistry Dashboard were derived for the individual chemicals. The effect of chemical-to-chemical interactions on protein binding efficiency of the individual chemicals also remains unclear. Third, the technologies used to derive protein binding values were different. Traditionally, rapid equilibrium dialysis is used to determine the protein binding efficiency of environmental chemicals and drugs. However, lipophilic chemicals may fail to reach equilibrium during dialysis, which in turn would underestimate the actual free fractions in the sample (Ferguson *et al.*, 2019). Instead, the use of SPME may provide more relevant estimates for the protein binding effects of lipophilic chemicals such as polycyclic aromatic hydrocarbons (logP = 3.3–6.8).

We note several limitations in this study. First, we performed a semiquantitative analysis using relative abundance to procure chemical profiles of aliphatic and aromatic compounds in petroleum substances. Semiquantitative analysis may lead to analytical bias from absolute concentrations for individual chemicals. Nevertheless, semiquantitative analysis can be a useful tool to investigate the chemical profiles and compositional similarity assessments (Grimm *et al.*, 2017) of complex substances. Second, only 53 aliphatic and aromatic compounds have been investigated in this study. Follow up studies using untargeted analyses would be beneficial to further characterize the overall chemical profiles of petroleum substances. Third, among the 25 tested aromatic compounds, only 1 compound (dibenzothiophene) belongs to ArS molecular class. Increasing the number of ArS compounds would enhance the representativeness of ArS molecular class. Finally, we used petroleum substances to represent the UVCBs. Other UVCBs may behave differently with petroleum substances. More case studies in this field would raise the overall confidence and acceptance of using *in vitro* data for the health and environmental assessments of UVCBs.

Overall, this case study used a defined mixture of analytical standards and 4 representative petroleum substances from different refinery streams to demonstrate that extraction procedures, protein binding in cell culture media and dilution factors prior to *in vitro* testing can all contribute to determining the final bioavailable concentrations *in vitro*. Thus, IVIVE for UVCBs may require greater attention to concentration-dependent and compound-specific differences in recovery and bioavailability.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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