CHARACTERIZATION OF COMPLEX SUBSTANCES USED IN BIOLOGICAL PROFILING THROUGH DETERMINATION OF THE FREE CONCENTRATION WITHIN IN VITRO ASSAYS

A Thesis

by

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ABSTRACT

Application of traditional high through-put (HTP) in vitro screening, coupled with advanced mass spectrometric and separation techniques, to substances of unknown or variable composition, complex reaction products, and biological materials (UVCBs) has made monumental progress toward biological and analytical profiling, facilitating regulatory read across and decision making for these challenging substances. However, limited understanding of protein and non-specific binding effects to the chemical composition of screening extracts may cause misinterpretations of potential in vivo effects. Conventional in vitro to in vivo extrapolation (IVIVE) approaches could alleviate these concerns, but current techniques only evaluate single-constituents. Furthermore, applied in vitro pharmacokinetic assays measuring protein binding, specifically equilibrium dialysis, are limited to hydrophilic chemicals.

This study clearly defines chemical in vitro equilibrium dialysis assay limitations through application of environmental chemicals with a range of octanol/water partition coefficients ($logK_{OW}$) and identifies solid phase micro-extraction (SPME) as a suitable alternative for environmental chemicals not suitable for equilibrium dialysis. This alternative technique is used to characterize the chemical composition of complex substances, used in biological profiling, by evaluating chemical protein binding of these substances within cell medium.

Lastly, this study discusses the utility of traditional chemical composition analysis, via GC-MS, to effectively group or “fingerprint” complex substances,

specifically petroleum products, for regulatory read across application. This is accomplished by evaluating its ability to effectively group similar petroleum refinement classes compared to more advanced analytical techniques (GCxGC-FID and IM-MS).

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NOMENCLATURE

AC_{50}	Active Concentration 50 percent
ADME	Absorption Distribution Metabolism and Elimination
ArS	Aromatic Sulfur bearing
C_{SS}	Concentration Steady State
DiAr	Di-Aromatic
DiN	Di-Napththenic
FMI	Fowlkes-Mallows Index
GC-MS	Gas Chromatography Mass Spectrometry
GCxGC-FID	Gas Chromatography by Gas Chromatography Flame Ionization Detection
HPLC-MS	High Performance Liquid Chromatography Mass Spectrometry
HTP	High Through Put
IM-MS	Ion Mobility Mass Spectrometry
Iso-P	Iso-Paraffin
IVIVE	In Vitro to In Vivo Extrapolation
LC-MS	Liquid Chromatography Mass Spectrometry
$LogK_{OW}$	Octanol/Water Partition Coefficient
NDiAr	Naphthenic Di-Aromatic
N-P	N-Paraffin
OED	Oral Equivalent Dose

PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffer Saline
PolyAr	Poly-Aromatic
PPB	Plasma Protein Binding
REACH	Registration Evaluation Authorization and Restriction of Chemicals
RED	Rapid Equilibrium Dialysis
SPME	Solid Phase Micro-Extraction
TriAr	Tri-Aromatic
UCM	Unresolved Complex Mixture
UVCB	Unknown or Variable composition Complex reaction products or Biological materials

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1. INTRODUCTION

Within the past decade new legislative actions, both within the United States and European Union, enacted new regulatory requirements in regard to chemical product registration and human health safety risk evaluations for chemicals currently used in commerce.[1,2] These new government regulatory provisions, Frank R. Lautenberg Chemical Safety for the 21st Century Act (US legislation) and Registration, Evaluation, Authorization and Restriction of Chemicals (REACH, EU legislation), mandated chemical manufactures to provide human health hazard information as part of new product registration requirements. Additionally, these regulations sought to close the health hazard information gap for thousands of chemicals currently used in commerce by applying specific deadlines for chemical manufactures and regulatory agencies to evaluate chemicals based on production or import quantities.[1,2] Both pieces of legislation retained the ability to conduct read across assessments, justifying chemical similarities, based on physicochemical properties, functional groups or biological response, between data poor and data rich chemicals.[1,2,3,4] As a result, a great demand exists to further facilitate grouping and read-across, through the application of biological and chemical compositional profiling, for unique chemical formulations and complex substances.

In order to meet health hazard evaluation deadlines, regulatory agencies focused on novel in vitro applications to accommodate initial risk prioritization for tens of thousands of chemicals used in today's markets, lacking sufficient health assessment information.[5,6] Traditional chemical toxicity evaluation incorporates the use of animal models comprising of acute, chronic, and long term cancer exposure assays to fully characterize a chemical's health risk assessment. These

models are impractical to evaluate over ten thousand chemicals, due to the associated time, labor, and financial burden. Instead, the United States Environmental Protection Agency (US EPA) utilized 21st century high throughput (HTP) in vitro screening approaches, through application of a suite of toxicodynamic assays, to perform first tier chemical hazard prioritization based on a chemical's potency to elicit an abnormal biological response.[5,6] Further EPA studies applied in vitro to in vivo extrapolation (IVIVE) by incorporating toxicokinetic parameters, distribution (evaluating plasma protein binding via rapid equilibrium dialysis (RED)) and metabolic clearance (measuring chemical disappearance via hepatic clearance). As a result, determination of a chemical's exposure oral equivalent dose (OED) facilitated a more comprehensive chemical risk prioritization for regulatory decision makers.[6]

However, these toxicokinetic assays, were designed and validated for pharmaceutical evaluation.[4,8] In general, most pharmaceuticals are designed to retain a degree of water solubility, maintaining an octanol/water partition coefficient ($\log K_{ow}$) lower than 5, enabling oral administration.[9] Environmental chemicals are not design under such restrictions, limiting their water solubility and potentially leading to systematic error within in vitro assay designs, namely plasma protein binding via the RED device. Furthermore, traditional in vitro toxicodynamic assays do not account for non-specific binding (NSB) of the test chemical to the walls of the testing plate, nor the effects of cell medium protein binding during the course of the study. These parameters affect the overall concentration of the chemical during analysis, increasing uncertainty within in vitro dose response evaluation.[7] Lastly, the current IVIVE design only evaluates mono-constituent substances with limited application toward mixtures or complex substances.[5,6]

Recently, several studies aimed to reduce the hazard assessment gap for mixtures and complex substances, by applying in vitro toxicodynamic assays along with enhanced analytical instrumentation to biologically profile these substances, specifically petroleum in order to facilitate grouping and regulatory read across.[3,4,10] Various forms of petroleum products are extensively used within modern commerce; over 10,000 tons manufactured or imported a year within the European Union and United States.[3,4] Consequently, most petroleum product registrations petitioned for some form of read across to meet the 2010 REACH regulatory deadlines.[4] Despite application of novel toxicodynamic profiling and exploitation of modern analytical chemical instrumentation, chemical regulators have rejected most read-across applications, citing uncertainties regarding chemical composition.

This study aims to reduce these uncertainties in several ways. First, in vitro equilibrium dialysis assay limitations will be clearly delineated through application of "no protein" equilibrium dialysis controls to test a variety of pharmaceuticals and environmental chemicals, spanning a range of octanol/water partition coefficients ($\log K_{OW}$). Subsequently, this study will evaluate solid phase micro-extraction (SPME) as a suitable alternative for environmental chemicals not applicable for equilibrium dialysis. Next SPME will be used to characterize complex substances used in toxicodynamic profiling, via measurement of the free concentration of chemicals within in vitro cell medium. Lastly, this study discusses the utility of traditional compositional analysis, via GC-MS, to effectively group or "fingerprint" complex substances, specifically petroleum products, for regulatory read across application. This is accomplished by evaluating its ability to effectively group similar petroleum refinement classes compared to more advanced analytical techniques (GCxGC-FID and IM-MS).

2. LITERATURE REVIEW

2.1 Introduction

Within the framework of chemical safety regulation, understanding how a chemical travels throughout the body (pharmacokinetics, toxicokinetics) and the characteristic effects the chemical imposes on the body's receptors, cells, and tissues (pharmacodynamics, toxicodynamics) provides the foundation toward a chemical's hazard identification and heath risk assessment. Traditionally, this information is obtained through animal models, utilizing individual chemicals, and bringing with them a significant time, labor, and monetary burden. As a result, a large effort has been made to incorporate in vitro assays into chemical health assessment procedures, designed to produce similar biological information, but increasing chemical capacity, at a fraction of the time, labor, and money compared to animal models. This application of these cellular or "cell free" assays sparked a demand to model the information to a biologically relevant mammalian system (in vitro to in vivo extrapolation ((IVIVE)), translating analysis into an easily quantifiable value (oral equivalent dose (OED)) for regulatory decision making and health risk assessment.

However, the majority of these assessments evaluate single chemicals, even though the bulk of registered substances are mixtures of chemicals or complex substances, products comprised of a multitude of different elements. This creates a challenge for chemical health evaluation. Consequently, a great effort has been focused on grouping various complex substances, based on similar analytical chemical characteristics (chemical fingerprinting) and biological response profiles, facilitating regulatory read across evaluation of products with limited health information to chemicals that are more understood. The following literature

review expands upon the previously mentioned techniques and subjects, highlighting their application toward chemical regulation and health risk assessment.

2.2 Pharmacokinetics and toxicokinetics

Pharmacokinetics have been applied by pharmaceutical industries and clinicians within the area of drug development, dosage regimens, routes of administration, and treatment duration to insure the administered drug achieves therapeutic effects for patients.[11] Likewise, chemical regulators apply the same processes, toxicokinetics, in order to characterize hazard identification along with exposure in chemical risk assessment.[6] Four pharmacokinetic properties; absorption, distribution, metabolism, and elimination (ADME) define how the body acts upon a chemical. When pharmacokinetic evaluation is applied in parallel with pharmaco-toxicodynamics, (the measure of how a chemical acts upon receptors, cells, or tissues in the body), researches are able to model a chemical's initiation, potency, and effect duration within the body.[11]

The pharmaceutical industry established the foundation of these kinetic parameters through application of pharmacokinetic computational modeling, used extensively within new drug development.[12,13] Modeling predictions of pharmacokinetic outcomes decreased failure rate within new drug development process and decreased the number of studies needed for approval.[12] Incorporation of in vitro assays, capable of providing concordant data with physiological parameters, contributed to the development of robust multiple compartment models, reducing uncertainty within modeling predictions as well as the time, labor, and cost associated with traditional animal models.[7,13] In vitro assays measuring plasma protein binding, through equilibrium dialysis, ultra-filtration, or solid phase micro extraction techniques, provide a validated application to determination of a chemical's unbound fraction in order to estimate its bioavailability and distribution throughout the body.[5,6,14] Additionally, application of cyro-

preserved human hepatocytes provided an accurate in vitro metabolic representation used to estimate in vivo human metabolic clearance.[15,16] However, these advances within in vitro testing remained centered in the pharmaceutical industry with limited application toward environmental chemicals.[5,6]

Within the last few years legislative initiatives within Europe and the United States revised existing requirements for chemical safety testing and registration, requiring regulatory agencies to develop more robust and inexpensive risk prioritization methods for thousands of chemicals currently used in commerce.[2,5,6] As a result, a battery of in vitro assays were developed to prioritize environmental chemicals based on their concentration at 50% maximum bioactivity, or AC_{50}.[5,6,7] However, without incorporation of a chemical's bioavailability, metabolic characteristics, and estimated real world exposure concentrations, this prioritization method displayed the potential to over or under estimate a chemical's risk toward human health.[5,6] Recent application of in vitro plasma protein binding, hepatic clearance assays, and the incorporation of in vitro to in vivo extrapolation (IVIVE) modeling, enabled regulators to derive dosimetry values, oral equivalent dose (OED), needed to reach bioactive blood concentrations.[5,6] Comparing the chemical's OED to real world estimated chronic exposure values provided a more powerful prioritization tool for chemical regulation.[5,6]

2.3 In vitro to in vivo extrapolation (IVIVE)

At the turn of the century scientists sought to leverage a multitude of in vitro toxicological assays designed to identify a chemical's potency to generate an abnormal biological response when applied to cells in culture at increasing concentrations. Increasing demand to prioritize thousands of environmental chemicals used in commerce, lacking human health safety information, spurred the development of the TOXCAST program by the US EPA.

Designed as a battery of in vitro cellular or cell-free assays, recognized by their ability to produce reproducible and robust information of biological profiles for pharmaceuticals, these assays provided the ability to conduct high throughput screening of thousands of chemicals.[5,6] However, correlating biological effects of cells in a dish to activities with an intact mammalian system remains a constant challenge for scientists and chemical safety regulators. As discussed earlier, evaluating a chemical's health effect on potency without considering toxicokinetic attributes can be misleading, limiting accurate risk prioritization. An IVIVE approach based on computational modeling of toxicokinetics was developed to apply information derived from in vitro assays to predict in vivo outcomes.[5,6] This approach predicts a steady state blood concentration through the in vitro measurement of ADME pharmacokinetic parameters. Pharmaceutical industries utilize this approach to determine therapeutic steady state blood concentration (Css) and the dose needed to achieve that blood concentration. Chemical safety regulators applied the same methodology to derive the following equation, using in vitro measurements to evaluate distribution (plasma protein binding) and metabolism (hepatic clearance), while incorporating conservative estimations for remaining absorption and elimination parameters.

$$C_{SS}=\frac{k_0}{(GFR \text{ x } F_{ub})+\left(\frac{(Q_1 x F_{ub} x Cl_{int})}{(Q_1+F_{ub} x Cl_{int})}\right)}$$

Each variable within the equation represents a specific physiological parameter. Where k_0 equals intake rate or rate of absorption, set at 1mg/kg·day.[5,6] F_{ub} equals the unbound fraction of parent chemical, measured through in vitro plasma protein binding. Cl_{int} equals intrinsic hepatic clearance, measured through in vitro hepatic clearance and scaled up to human physiological values. GFR equals glomerular filtration rate and Q_1 equals liver blood flow, set at a constant

human physiological values through the assumption that elimination is restricted by hepatic metabolism and renal filtration.[6] An OED is determined by applying reverse dosimetry to determined exposure levels needed to reach steady state blood concentrations of in vitro toxicodynamic active concentration 50 percent (AC_{50}) values.[6]

$$\text{Oral equivalent dose(mg/kg/day)} = \text{ToxCast } AC_{50} \text{ or LEC}(\mu M) \text{x} \frac{1\text{mg/kg/day}}{C_{ss}(\mu M)}$$

Appling IVIVE, regulatory studies assumed each chemical would have 100% absorption through the gut, resulting in 100% bioavailability. Additionally, it is estimated elimination would be limited to renal filtration and restrictive hepatic metabolism, and only the unbound chemical fraction was available for metabolism and elimination. Additionally, researchers assumed no active renal or biliary re-absorption and did not measure the formation of metabolites, only the disappearance of parent compound, estimating bioactivity resulted from exposure of the parent chemicals alone.[5,6] Overall, these assumptions retained a protective stance toward IVIVE modeling predictions.[5,6] Errors within these assumptions such as over estimation of absorption or under estimation of clearance, would lead to an overestimated C_{ss}, resulting in a more protective OED estimation. While not ideal, this limitation is more favorable than the latter when applied to human health risk assessment.[6] Appling additional in vitro assays and analytical techniques would lower the likelihood of prediction errors.[5,6]

Conservative estimations are not limited to the IVIVE mathematical model. Regulators must assume in vitro pharmacodynamic AC_{50} values would produce similar biological responses within a mammalian tissue. However, underlining uncertainties reside within these AC_{50} values. Binding to proteins within cell culture media, non specific binding to the in vitro plate, and chemical solubility potentially alter the actual bioactive concentration of chemical exposed to

cells in culture. These values are rarely analyzed due to the HTP nature of the in vitro assays. Lastly, the overwhelming majority of pharmacokinetic and pharmacodynamic assays limit analysis to individual chemicals without considering mixtures or complex substances expressed as UVCBs (Unknown or Variable composition, Complex reaction products or Biological materials).

2.4 Grouping and read-across used in biological profiling of complex substances (UVCBs)

In June of 2007, the European Union enacted legislation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).[1] As a result, all chemicals manufactured or imported into the European Union in excess of 1000 ton/year were required to meet new product registration requirements by 2010.[1,3] The REACH legislation required a robust range of assessment factors to be evaluated on an individual substance basis covering potential health effects, routes of exposure, and susceptible populations in order complete chemical safety assessment criteria.[1,3] Petroleum products display a unique problem set, lacking broad human health data and varying in chemical composition by different refinement processes, designed to meet desired performance criteria rather than uniform composition. As a result, more than 8000 individual petroleum products applied a form of read-across, grouping substances based on similar chemical structure, functional groups, and biological effects, to fill data gaps needed to meet registration health assessment requirements.[1,3] Described in Annex XI of REACH legislation, read-across requires justification to established acceptable similarity between data poor and data rich substances.[1] Moreover, petroleum substances were further classified by name, carbon chain length, viscosity, carbon cut-off , and boiling point values. Applying a chromatograph as a substance fingerprint was also used to characterize the composition of the petroleum substance.[1] As a result, traditional and novel analytical separation techniques have

been applied to a range of petroleum products in order to comprehensively categorize petroleum substances based on their refinement process and compositional content.[4] Additionally, novel in vitro toxicodynamic applications have been applied to petroleum products, at varying stages of refinement, to establish a biological profile in order to decreases the information gap, facilitating more accurate and precise read-across categorization for future complex substance registration.[4]

2.5 Chemical fingerprinting of petroleum substances through analytical chemistry

The methodology of petroleum fingerprinting stems from analytical procedures developed by petroleum geochemists in the 1970s.[17] In the pursuit of petroleum exploration and product application, geochemists evaluated different petroleum substances based on n- alkane boiling point profiles.[17] This basic technique is still used by petroleum industries as a first tier evaluation of the petroleum refinement process toward desired performance specifications. Moreover, petroleum fingerprinting has also been extensively used within the environmental protection field to measure petroleum biomarkers, evaluate oil weathering in oil spill analysis, and forensically determine the origin of petroleum contamination.[17]

The predominate analytical instrument used to fingerprint petroleum substances is the Gas chromatography mass spectrometer (GC-MS). Generally, a GC-MS instrument employs a capillary column, heated by an oven at a predetermined temperature gradient in order to separate compounds by boiling point and polarity. The eluting compounds are then ionized and analyzed by a detector. Since molecules of specific molecular classes maintain distinct mass ion fracture patterns, a GC-MS is able to differentiate ion signals from multiple compounds. However, a GC-MS instrument's column peak capacity can become overloaded, causing a baseline hump termed as an unresolved complex mixture (UCM). In this instance, the column no longer has the resolving power to separate all the compounds within the sample. This is typically seen in

petroleum analysis, since an individual petroleum substance contains more than 10,000 different chemical compounds.[17] This limits the amount of molecules the instrument can effectively differentiate, hindering its ability to produce a robust chemical fingerprint.

In recent years, instrument resolving power and sensitivity has increased, allowing for more detailed characterization of complex substances. The incorporation of two gas chromatography columns with different selectivity (GCxGC-FID) increases the peak capacity of the instrument and allows for improved separation of molecules that form a UCM under GC-MS analysis.[18] Moreover, ion mobility mass spectrometry (IM-MS) incorporates unique ionization methods, electron spray (ESI) or atmospheric photo ionization (APPI), along with separation techniques based on size, shape, and charge of the ionized molecule. This further increases analytical sensitivity and enables improved chemical fingerprinting. Lastly, fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) increases resolution and mass accuracy, enabling the acquisition of specific structural information enhancing petroleum characterization.[17]

Albeit, these instruments enhance the ability to characterize complex substances like petroleum products, their application is still novel and not widely understood within the scientific, regulatory, or industry communities. Additionally, these instruments are complex and costly, limiting access to the instruments.[19] Moreover, the majority of regulatory and standardized chemical compositional analysis protocols utilize GC-MS as the instrument of choice.[17,19] As a result, GC-MS remains the primary workhorse for petroleum chemical fingerprinting.

3. MATERIALS AND METHODS

3.1 Utility of the rapid equilibrium dialysis (RED) assay

3.1.1 Chemical selection, reagents and stock preparation

Phosphate buffer saline (PBS), LC-MS grade acetonitrile, dimethyl sulfoxide (DMSO), LC/MS grade water with .1% formic acid, and LC-MS grade methanol were purchased from Fisher Scientific (Waltham, MA). Pharmacueticals: Propranolol, sotalol, and isoproterenol were purchased from Molecular Devices (Sunnyvale, CA). Cisapride monohydrate was purchased from Sigma-Aldrich chemicals (St. Louis, MO). These pharmaceuticals were selected because they have been extensively studied and cover a range of plasma protein binding values as well as octanol/water partition coefficients' ($\log K_{OW}$). Environmental chemicals: carbaryl, pirimicarb, permethrin, acenaphthene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, and phenanthrene were purchased from Sigma-Aldrich chemicals (St. Louis, MO). Prometon was purchased from Accustandard (New Haven, CT). Acenaphthene, carbaryl, permethrin, pirimicarb, and prometon have been evaluated with the RED device in other studies and were selected to assess reproducibility of the RED device.[6] Remaining environmental chemicals were selected to evaluate $\log K_{OW}$ effects within the RED device. In total, all selected chemicals cover a broad spectrum of $\log K_{OW}$ values, ranging from .1 (isoproterenol) to 6.75 (dibenz(a,h)anthracene) (Table 1). All chemicals and reagents were stored according to manufacture guidelines. Pharmaceuticals and environmental chemicals were purchased in neat form and diluted in 100% DMSO to working stock concentration of 2mM and stored at $< -70^0$C until use.

3.1.2 Plasma protein binding via RED assay

Plasma protein binding was evaluated for each chemical utilizing the rapid equilibrium dialysis (RED) method as described in other publications, but modified to incorporate no protein equilibrium controls (Figure 1).[6,8] Human plasma was recovered from whole blood donations using anti-coagulant (K_2EDTA) and pooled from healthy donors at a U.S. Food and Drug Administration- licensed donor center (HMPLEDTA2; Bioreclamation, Inc., Westbury, NY). All donors tested negative for HIV ½ AB and HCV AB and non-reactive for HBSAG, HIV-1 RNA, HCV RNA, HBV DNA and STS.[6,8] Prior to analysis, human plasma, stored at $< -70^0$C, was thawed to room temperature and centrifuged at 2000 X g for 10 minutes to remove particulates.[6,8] The RED assay was conducted using RED inserts (catalog no. 90006, Pierce Biotechnology, Rockford, IL) according manufacture instructions, with protocol modification to incorporate protein free equilibrium controls. Equilibrium controls comprising of PBS buffer in both sample and buffer chambers were designed to ensure pharmaceuticals fully equilibrated within the device in the absence of proteins (Figure 1). DMSO chemical stock solutions were diluted 200 fold in human plasma to test concentration of 10µM. Final DMSO concentration did not exceed 0.5%. Sealing tape was placed the RED device and it was incubated at 37^0C for 4 hours at 100 oscillations per minute on an orbital rocker.[6,8] Upon completion of incubation, 50µL aliquots were removed from each chamber and matrix matched with equal volumes of plasma, or buffer. Samples were diluted with 300µL 100% acetonitrile and frozen at $< -70^0$C until analysis.[6,8] Aliquots of spiked human plasma, and PBS working stock solutions were removed to measure percent recovery. These percent recovery samples followed the same matrix match and acetonitrile dilution pattern. All RED assays were completed in triplicate.

3.1.3 RED assay plasma protein binding calculation

Chemical unbound percentages were calculated by measuring the concentration within both chambers, sample and buffer. The concentration in the buffer chamber was then divided by the concentration detected in the sample chamber and multiplied by 100 to determine the percent unbound value. Triplicate percent unbound values were averaged to determine the final unbound value. No testing concentrations were below the analytical detection limits.

3.1.4 Analysis by high performance liquid chromatography with mass spectrometry detection

Analysis was performed by an Agilent 6470 triple quadrupole mass spectrometer (Santa Clara, CA) operating in positive ion mode with a Waters Acquity H class HPLC (Milford, MA). Chromatography separation was performed on a C_{18} column (Agilent Zorbex Eclipse Plus C_{18} 3.0 X 50mm, 1.8 mircron) with a C_{18} guard column (Santa Clara, CA).

Analysis of pharmaceuticals: Aqueous mobile phase consisted of .1% Formic Acid and acetonitrile for organic mobile phase. 10µL sample injections were separated using the following a solvent gradient: (1) 2% organic for 1 min; (2) linear gradient ramp to 95% organic over 1.5min; (3) 95% organic maintained for 1.5min; (4) linear gradient ramp to 2% organic over .2min; (5) 2% organic condition held for 3.8min until next injection. Total analysis time was 8 minutes at a flow rate of 400µL per minute.

Analysis of environmental chemicals: chromatography conditions followed a previously described method with slight modification.[6] Aqueous mobile phase consisted of .1% Formic Acid and methanol for organic mobile phase. 5µL sample injections were separated using the following a solvent gradient: (1) 20% organic for 0.5 min; (2) linear gradient ramp to 100% organic over 4.5 min; (3) maintain 100% organic for 1 min; (4) linear gradient ramp to 20%

organic over 0.5 min; and (5) maintain 20% organic for 2 min prior to the next injection. Total analysis time was 8.5 min per sample at a flow rate of 400µL per minute. All samples (environmental chemicals, and pharmaceuticals) were introduced to the mass spectrometer in splitless mode with an AJS ESI ion source. Complete mass spectrometry conditions for all chemicals are listed in (Table 2).

RED assays samples were thawed at room temperature, vortexed, and centrifuged at 12,000 X g for 10min. Supernatant was transferred to a separate vial and diluted 1:4 with .1% formic acid and water. Pharmaceutical samples were spiked with a known amount of internal standard, sotalol (CAS: 959-24-0), prior to analysis. Environmental chemical samples were spiked with a known amount of internal standard, isoxaben (CAS: 82558-50-7), prior to analysis. Calibration curves were created prior to analysis in an identical matrix to the samples.

3.1.5 Analysis by selective ion monitoring gas chromatography mass spectrometry detection

Analysis was performed by an Agilient 6890N gas chromatogram with a Agilient 5975C mass spectrometer (Santa Clara, CA) operating in electron impact ionization mode. Sample data was collected via selective ion monitoring mode, for additional instrument parameters see (Table 3). Chromatography separation was performed with 2µL spiltless sample injections on an Agilinet DB-5ms column (Agilent DB-5 30.0 X 250mm, .25 µm film thickness). Separation was performed using the following oven gradient: (1) initial injection port temperature set to 300^0C with initial oven temperature set to 60^0C; (2) Oven temperature increased 15^0C/min to 150^0C; (3) Oven temperature increased 5^0C/min to 220^0C; (4) Oven temperature increased 10^0C/min to 300^0C and held for 10min. Total run time was 38 minutes.

RED assays samples were thawed at room temperature, vortexed, and centrifuged at 12,000 X g for 10min. Supernatant was transferred to a separate vial for hexane extraction. 1ml of hexane

was added to each sample along with a know amount of internal standards (naphthalene-d_8 CAS 1146-65-2, acenapthene-d_{10} CAS 15067-26-2, phenanthrene-d_{10} CAS 1517-22-2, chrysene-d_{12} CAS 1719-03-5, perylene-d_{12} CAS 1520-96-3). Samples were vortex briefly and excess water was removed by addition of sodium sulfate (Na_2SO_4). Hexane was transferred to a separate vial and remaining Na_2SO_4 was rinsed three times with 1ml of hexane. Hexane extract, approximately 5ml, was transferred to 25 ml glass concentrator tubes and submerged into a hot water bath. Extracts were concentrated to approximately 200µL prior to analysis. Calibration curves prepared in hexane were created prior to analysis.

3.2 Chemical characterization of complex substances (petroleum)

3.2.1 Materials.

iCell cardiomyocyte maintenance medium was purchase form Cellular Dynamics (Madison, WI). William's E Medium (no phenol red), clear bottom plastic, and glass coated 384 well plates were purchased from Fisher Scientific (Waltham, MA). Samples of petroleum substances from four separate refinement processes, straight run gas oils (SRGOs, n=5), other gas oils (OGOs, n=2), vacuum and hydrotreated gas oils (VHGOs, n=8) and heavy fuel oils (HFOs, n=3) were provided by Concawe (Brussels, Belgium). SPME LC C_{18} fibers were purchased from Sigma-Aldrich chemicals (St. Louis, MO). Polycyclic-aromatic hydrocarbon (PAH), saturated hydrocarbon, and crude oil standards were provided by The Texas A&M Geochemical and Environmental Research Group (GERG) (College Station, TX).

3.2.2 Petroleum and analytical standard DMSO extract preparation

DMSO extracts of petroleum products have been use as a method to conduct dermal toxicity tests as well as with in vitro applications.[4,10] Extract preparation has been described elsewhere.[4,10] Briefly, 4g of each petroleum product was dissolved in 10ml of cyclohexane and

extracted twice with 10ml of pre-equilibrated DMSO/cyclohexane (10:1) solution. An analytical standard extract was prepared in the same manner by spiking 10ml of cyclohexane with a known amount of PAH and saturated hydrocarbon standard solutions, for complete standard listing see (Table 4). Each stock petroleum extract was diluted 100 fold with pure cell culture grade DMSO yielding two working concentrations (100% and 1%). These pure DMSO working concentrations were subsequently diluted 200 fold in cell culture media (icell cardiomyocyte maintenance media containing a standard amount of bovine serum albumin (BSA) or William's E medium containing no serum proteins), yielding two testing solutions (.5% and .005% of stock DMSO extract) with 0.5% total DMSO content. One petroleum sample from each manufacturing stream (OGO, SRGO,VHO, HFO) was used throughout the course of this study. A single analytical standard DMSO extract concentration, following the same cell media dilution pattern, was utilized during this study. Samples were transferred to glass coated and plastic 384well plates (n=3 per petroleum sample per plate). One set of plates (glass and plastic) was immediately frozen at <-70^{0}C (Time point =0hrs) until analysis. Another set of 384 well plated was incubated at 37^{0}C for 4hrs (time point =4hrs) then frozen at <-70^{0}C until analysis.

3.2.3 Media protein binding analysis via solid phase micro-extraction (SPME)

Protein binding analysis follows previously described methods with some modifications[20,21] LC C_{18} SPME Fibers were preconditioned in methanol/Milli-Q water solution (50:50) according to manufacture instructions. iCell cardiomyocyte maintenance medium was thawed to room temperature and penicillin-streptomycin was added according to manufacture instructions. Petroleum extract samples, two concentrations diluted into cell media as described above, were place into 2ml amber glass vials containing 200µL glass inserts. Total sample volume was 100µL and analyses were performed in triplicate. Prior to SPME fiber extraction,

samples were allowed to equilibrate on an orbital shaker (500rpm) under incubation for 1hr. After equilibration SPME fibers were inserted through the vial cap septa and placed in the incubator on an orbital shaker (500rpm) for 3hrs. After the 3hr allotment, SPME fibers were removed, rinsed briefly with Milli-Q water and placed in 100µL of desorption fluid consisting of 100% acetonitrile. Fibers were placed on an orbital shaker (500 rpm) and desorbed for 30min at room temperature. Standard solutions were prepared in PBS, following the same dilution patterns and fiber extraction, desorption procedures as previously mentioned. SPME protein binding controls (propranolol, acenaphthene, and permethrin) were tested at 10µM concentrations in pooled human plasma, prepared in the same manner as the RED assay. These control chemicals were incorporated in order to validate the SPME method's ability to produce accurate and precise protein binding data.

3.2.4 Calculation of SPME protein binding

Determination of unbound chemical concentrations using SPME followed procedures outlined in published literature.[20] Briefly, the fiber constant (f_c), representing the partition coefficient between unbound chemical in solution and the amount of absorbed to the fiber, was determine by analyzing standard solutions of chemical in PBS.

$$f_c=\frac{C_{e,s}}{C_{0,s} - C_{e,s}}$$

Where $C_{0,s}$ is the initial concentration prior to fiber extraction and $C_{e,s}$ represents the concentration of the chemical extracted by the fiber. When SPME is performed in a sample containing proteins and concentration of the chemical is extracted by the fiber (C_e). The unbound concentration (C_{free}) in the sample is determined using the following equation.

$$C_{free}=\frac{C_e}{f_c}$$

The final total concentration (C_t) of chemical in the sample is determined using the following equation, where C_0 represents the initial chemical concentration prior to fiber extraction.

$$C_t = C_0 - C_e$$

Ultimately, the percentage unbound (% Unbound) is calculated from the total and free concentration of the chemical as displayed below.

$$\% \text{ Unbound} = \left(1 - \frac{C_t - C_{free}}{C_t}\right) x100$$

3.2.5 Sample analysis by selective ion monitoring gas chromatography mass spectrometry

Analytical method was designed in accordance with ASTM D5739 with some modification.[25] Analysis was performed by an Agilent 6890N gas chromatogram with an Agilent 5975C mass spectrometer (Santa Clara, CA) operating in electron impact ionization mode. Sample data was collected via selective ion monitoring mode, for additional instrument parameters see (Table 5). Chromatography separation was performed with 1µL spiltless sample injections on an Agilinet DB-5ms column (Agilent DB-5 30.0 X 250mm, .25 µm film thickness). Separation was performed using the following oven gradient: (1) initial injection port temperature set to 250^0C with initial oven temperature set to 55^0C; (2) Oven temperature increased 6^0C/min to 270^0C; (3) Oven temperature increased 3^0C/min to 300^0C; (4) Final oven temperature of 300^0C held for 17min. Total run time was approximately 65 minutes.

Pure petroleum DMSO extracts, 384 well plate samples, and SPME samples were diluted 1:2 with 4% sodium chloride solution and extracted two times with 2ml and 1ml of pentane. Excess water and DMSO was removed through addition of Na_2SO_4. Pentane extract was transferred to a separate vial and remaining Na_2SO_4 was rinsed three times with 1ml of pentane. Pentane

extracts were transferred to 25 ml glass concentrator tubes and submerged into a hot water bath. Extracts were concentrated to approximately 100µL prior to analysis. Semi-quantitative analysis was performed through integration of total 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 two dimensional matrix, evaluating the percent composition of the compound in relation to 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 two dimensional matrix example see (Table 6).

Neat petroleum substances were analyzed in a similar fashion as described above with the exception of performing a 1:500 spilt injection and conducting a full scan of ion mass ranging from 55-300 m/z for full instrumental parameters see (Table 5). Neat petroleum samples did not go through any solvent preparation prior to GC/MS split injection.

4. RESULTS

4.1 RED utility analysis

An initial utility analysis evaluated chemical aqueous solubility, specifically octanol/water partition coefficient ($LogK_{OW}$), to RED derived plasma protein binding values in published IVIVE literature.[6] Multivariate analysis, across 384 environmental chemicals, yielded a significant correlation between a chemical's $LogK_{OW}$ and its unbound percentage, indicating greater $LogK_{OW}$ produce lower unbound percentage within the RED device (Figure 2A). An assortment of 13 chemicals, comprising of pharmaceuticals, agricultural chemicals, and industrial chemicals, with $LogK_{OW}$ values ranging from less than 1 to greater than 5 (Figure 2B) were selected to validate reproducibility of the RED device and confirm aqueous testing limitations. Measured unbound values for 4 of the 5 environmental chemicals (pirimicarb, prometon, acenaphthene, and permethrin) corresponded well to RED assay literature values (Figure. 3A). However, carbaryl yielded a lower value of 12% unbound compared to a literature value of 70% unbound,[6] displaying a more highly bound characteristic similar to pirimicarb and other carbamate insecticides.[23] When these 5 chemicals were applied to PBS equilibration controls, a reduction in equilibration was observed for acenaphthene and phenanthrene with $LogK_{OW}$ 3.92 and 4.46 respectfully. Alternatively, permethrin, yielding a $LogK_{OW}$ of 6.5, failed to equilibrate within the PBS control (Figure 3B).

Evaluated pharmaceuticals with $LogK_{OW} <5$ (cisapride, propranolol, and isoproterenol), displayed unbound characteristics consistent with drug label references with cisapride being the most highly bound and isoproterenol displaying the largest unbound percentage. Isoproterenol RED assay results provided an unbound percentage of 52% compared to 35% from a historical

study conducted in the 1970s with thyroid patients.[24] No other recent studies were found for comparison (Figure 4A). All pharmaceutical successfully equilibrated within the RED device in PBS controls (Figure 4B).

Remaining 4 of the 13 validation chemicals, consisting of polycyclic-aromatic hydrocarbons (PAH) with $LogK_{OW} > 5$, yielded highly protein bound percentage (Figure 5A). However, all of these chemicals failed to equilibrate within the PBS controls (Figure 5B), raising uncertainty toward the measured unbound values. This observation underscores an important limitation for the RED device pertaining to hydrophobic environmental chemicals.

4.2 Chemical characterization of complex substances (petroleum)

4.2.1 Solid phase micro-extraction (SPME) technique validation

While recognized as the "gold standard" in determining chemical protein binding,[14] equilibrium dialysis is limited to hydrophilic chemicals. SPME techniques present a possible alternative to accurately measure protein binding for chemicals not suitable for the RED assay. The SPME device consists of small rods covered in a material that absorbs a fraction of the chemical in equilibrium with the sample's unbound concentration.[7,20,21] This technique has been utilized in a variety of applications; ecological contamination monitoring, in vitro protein binding modeling, to analysis of a variety of chemicals, ranging from pharmaceuticals to hydrophobic environmental chemicals.[7,16,20,21,25] Pilot chemicals, propranolol, acenaphthene, and permethrin, each spanning a range of $LogK_{OW}$ values (3.48, 3.92, 6.5) and sharing lipophilic characteristics, were utilized to validate the technique.[11,26] Measured unbound values for propranolol and acenaphthene correspond well to literature references utilizing the RED device (Figure 6A).[6,8,20] SPME unbound concentration of permethrin yielded a 70 fold increase compared to the RED device (Figure 6B). Notably, the SPME derived unbound value

corresponded well to values observed from a protein binding study measuring ^{14}C- labeled permethrin recoveries through a 3 phase organic solvent extraction to isolated bound and unbound concentrations (Figure 6C).[26]

4.2.2 Petroleum DMSO extract analysis

Evaluation of DMSO extraction efficiency utilizing PAH and Aliphatic hydrocarbon analytical standards, displayed high extraction efficiency for PAH compounds, resulting in over 70% recovery from initial spike concentrations (Figure 7). However, DMSO was not effective at extracting the more non-polar straight or branched aliphatic hydrocarbons, yielding recoveries less than 10% (Figure 7A, 7C). Consequently, DMSO enables isolation of the Aromatic Ring Class (ARC) compounds from a petroleum substance, generally considered the most toxic element of petroleum substances.[4] This extraction characteristic is replicated through compositional analysis of neat and extracted petroleum products from all four different refinement streams (SRGO, OGO, VHGO, HFO) evaluated within this study.

Linear alkanes (n-paraffins) constituent for the majority of molecular components within neat gas oils, (SRGO, OGO, VHGO) with each refinement stream displaying a variety of aromatic content (Figure 8). Alternately, HFO substances are manufactured from the residue of the pervious gas oil distillates,[4] resulting in larger quantities of higher molecular weight molecules, reducing the total amount of n-paraffins (n-P) and iso-paraffins (iso-P) and increasing the amount of aromatic compounds (Figure 8D). Visual comparison of the DMSO extract's chemical composition to the "neat" parent substance yields a significant change in the substance's chemical signature. More non-polar compounds; linear, branched alkanes (n-P and iso-P), and cyclic alkanes (DiN) are transferred in limited quantities, while the more polar aromatic compounds transfer in greater abundance into DMSO, concentrating the overall

aromatic content (Figure 8). However, the extracted aromatic content maintains same unique relative abundance pattern ratio as seen in the neat substance (Figure 9). Albeit, the aromatic compounds concentrate within the extract, only a small fraction remains for in-vitro toxicity testing when compared to the neat substance (Figure 10). However, this reduction in quantity does not degrade the samples' ability to elicit cytotoxic observations at high dose concentrations within biological profiling in vitro assays.[4]

4.2.3 Evaluation of cell medium protein binding for complex petroleum substances

Cell medium protein binding was evaluated using SPME technique at two testing concentrations of petroleum extract samples. Logarithmic dilutions of petroleum DMSO extracts, one for each petroleum manufacturing stream (SRGO, OGO,VHGO, HFO), were performed within cell culture medium containing an unknown concentration of bovine serum albumin (BSA), producing testing concentrations consisting of 0.5% and 0.005% of the starting DMSO extract. Total DMSO content remained constant at 0.5% across all samples. This dilution method is consistent with a in vitro biological profiling study utilizing identical petroleum substances.[4] A comparative protein binding analysis of five aromatic molecular classes across all petroleum samples, suggests the majority of aromatic compounds remain unbound at the highest concentration (Figure 11). Di-Aromatic molecular classes displayed protein binding characteristics, in both high and low concentrations, across all four petroleum sample types. Whereas, the Naphthenic Di-Aromatic molecular classes yielded characteristic protein saturation signatures within the high concentration for all samples types (Figure 11). Larger aromatic compounds (3 rings or greater) remain unbound at both high and low concentrations (Figure 11). Protein binding analysis through the use of aromatic standards depicts a clear trend between the number aromatic rings and the unbound concentration, with

aromatic compounds containing 3 or more rings yield a greater unbound concentration (Figure 7D).

As previously discussed, non-polar linear, branched, and cyclic alkanes molecular classes transfer in very small quantities into DMSO, stressing the detection limits of the analytical instrument for these compounds. Furthermore, these molecular classes are extremely water insoluble, averaging water solubility less than 1 part per billion.[27] Consequently, accurate protein binding analysis is unfeasible within the parameters of this assay.

4.2.4 Non-specific binding (NSB) analysis across different in vitro plate designs

Comparison of NSB effects, between glass and plastic in vitro plates, through the use of analytical standards produced significant recovery correlations in both pure DMSO and cell culture medium without protein conditions, yielding no significant reduction in NSB effects across designs (Figure 12). However, recovery values were profoundly reduced within the cell medium in both plate conditions, characteristic of these compounds' low water solubility (Figure 12B).[27]

4.3 Evaluation of analytical instrumentation via grouping of petroleum substances

4.3.1Petroleum compositional analysis

Chemical compositional analysis was performed on 15 neat samples across three distinct petroleum manufacturing methods, using three different analytical instruments; GC-MS, GCxGC-FID, and IM-MS. Analyzed abundance values were normalized relative to the total abundance detected for each sample. Compounds were organized by molecular class and carbon number range, producing a two dimensional data matrix for each sample (Table 6). Compounds detected varied across instruments due to the diversity of analytical methods utilized and capabilities (resolution and sensitivity) of the instrument.

GC-MS analyzed 8 separate molecular classes, comprised of n-alkanes and polycyclic aromatic compounds, across carbon number ranges of 6 to greater than 33, producing a total of 192 compositional data points. GCxGC-FID analyzed 9 separate molecular classes, producing 240 compositional data points. IM-MS analyzed 8 molecular classes, producing 312 compositional data points. Notably, IM-MS ionization method, APPI, expanded aromatic and heteroatom detection capability, but significantly reduced the n-alkane profile compared to the other instruments.[4]

4.3.2 Grouping via unsupervised hierarchical clustering

Pearson correlation values were derived from compositional matrices across all 15 neat samples for each instrument. Average linkage hierarchical clustering was exploited to evaluate each instrument's ability to group samples according to their refinement process (SRGO, OGO, VHGO). Clustering efficiency of each refinement category varied across all analytical instruments, with VHGO displaying the largest distribution (Figure 13). This evidence attributes to the compositional similarity across all three refinement categories, creating a challenging task toward selecting the most effective instrument.

4.3.3 Clustering evaluation via fowlkes-mallows index

Visual evaluation of an instrument's grouping effectiveness is unfeasible due to inconsistent clustering results across all three instruments. Therefore, Fowlkes-Mallow index (FMI) was applied to rank each instrument's hierarchical clustering result, using the three refinement categories as a benchmark.[28] The FMI score ranks clustering results on a 0 to 1 scale, 0 (least similar) to 1 (most similar), to a standard reference, in this case the refinement categories. Post evaluation, all instruments displayed similar grouping efficiency, with no

instrument displaying a significant advantage. IM-MS scored the highest index value of .43 with GC-MS and GCxGC-FID scoring values of .36 and .34 respectfully (Figure 13).

5. DISCUSION, LIMITATIONS, CONCLUSION, AND FUTURE PROJECTS

5.1 Discussion

Determination of a chemical's unbound concentration plays a significant role in assessing its distribution throughout the body, and contributes to its rate of elimination via metabolism and excretion. Pharmaceutical industries and regulatory institutions utilizes this parameter to estimate bioavailability and safe dosage levels.[6,8,11,14] As a result, equilibrium dialysis is recognized as the "gold standard" within the pharmaceutical industries.[14] The incorporation of the RED device into pharmaceutical evaluation dramatically reduced time, labor, and data uncertainty common with other equilibrium dialysis methods.[8] Moreover, its ability to screen hundreds of chemicals, attracted regulatory institutions to incorporate the assay into environmental chemical health risk assessment.[5,6] However, to appropriately utilize the device researchers must understand the physical chemical properties of the evaluated chemicals and institute controls to ensure data accuracy.

This study illustrates that chemical water solubility represents a critical factor in determining the RED device's suitability toward accurate protein binding evaluation. The implementation of protein free controls through the evaluation of environmental chemicals spanning a range of water solubility ($LogK_{OW}$) reveals hydrophobic chemicals, $LogK_{OW}$ greater than 5, fail to fully equilibrate within the device, producing inaccurate data. This inaccuracy can create significant problems with regard to IVIVE and pharmacokinetic modeling by altering derived OEDs. This could create unwanted adverse effects for pharmaceuticals or anti-protective dose references within exposure risk assessment. Published regulatory IVIVE models take a health protective approach by matching C_{SS} with total blood concentration, as opposed to free

concentration. As a result, increasing the unbound chemical concentration contributes to a greater metabolic clearance, constituting to a higher OED, so the errors identified in this study lead to “conservative” estimates that tend to overestimate risks.[5,6] However, implementing alternative techniques to increase accuracy for hydrophobic environmental chemicals will improve current models and increase confidence within in vitro assays and IVIVE dose predictions for regulatory decision making.

The SPME technique implemented in this study demonstrates its suitability as an alternative method to evaluate in vitro protein binding of hydrophobic environmental chemicals. Moreover, SPME has additional advantages, such as increasing detection signal during chemical analysis by reducing instrumental noise commonly attributed to matrix effects, and reducing financial, time, and labor costs compared to the RED device. Lastly, this study highlights the technique’s ability to measure unbound concentrations within complex substances in order to supplement findings for in vitro biological profiling assays.

These innovative in vitro biological profiling assays have contributed an insightful metric toward strengthening regulatory read across assessments for UVCBs, reducing uncertainty for an increasingly problematic element for chemical manufactures and regulators conducting human health assessments.[4,10] However, due to the ambiguous nature of these UVCBs, regulators still raise concerns regarding the chemical composition of the extract utilized within the in vitro assays and its representativeness with respect to the final manufactured product. This study used advanced analytical chemistry techniques, measuring the relative abundance of chemical classes commonly used in petroleum “fingerprinting methods”, to characterize how a sample’s chemical profile changed through each step of the in vitro toxicity evaluation.[17,22] Analysis, across four refinement classes of petroleum, the petroleum extract and unbound chemical concentration

within cell medium suggests that the aromatic ring class (ARC) components of the petroleum product predominately generate the biological responses observed by in vitro assays. Furthermore at lower concentrations, the more water soluble ARCs (compounds with 2 aromatic rings) display a higher protein binding affinity, reducing their availability to cells, than less soluble ARCs (compounds with 3 or more aromatic rings). These cell medium protein binding effects are often unaddressed when interpreting results for toxicodynamic assays.[7] More importantly, understanding which compounds are more bio-available within a complex substance provides an additional opportunity to augment substance pattern analysis, facilitating a more comprehensive categorization of these challenging substances for regulatory read across.[1,4,10]

Molecular compositional pattern analysis is one of many analytical tools used to categorize similar substances for regulatory read across.[1] Specific to petroleum substances, there is a great demand to improve resolution and sensitivity of analytical instrumentation to facilitate more comprehensive characterization of a petroleum substance's diverse chemical profile.[4,19,29,30] This study analyzed petroleum chemical profile information from three separate instruments, spanning a range of sophistication in terms of instrumental resolution and sensitivity.[4,17,18,19,30] Each instrument had unique advantages and disadvantages toward effectively grouping neat petroleum samples according to their refinery process. First, IM-MS displays the highest degree of resolution and sensitivity through advanced separation techniques.[4,30] Additionally, its expedient data acquisition time, (minutes vs. hours) and ability to effectively group substances based on heteroatom profiles gives it a distinct advantage over GCxGC-FID and GC-MS instruments.[4] However, IM-MS ionization techniques fail to capture a petroleum substance's n-alkane profile, the major component of petroleum.[4] Furthermore, the cost of the instrument and infancy of the technology, limits access to the instrument within the scientific community.[17,19]

Second, incorporation of two chromatographic columns in series increases the instruments peak capacity and resolving power for GCxGC-FID. Additionally, the instrument has been adopted by many of the major petroleum industries and is gradually being incorporated into published regulatory and scientific protocols.[18] However, unlike IM-MS or GC-MS, GCxGC-FID has no molecule specific ionization pattern detection capability, making it difficult to accurately define individual chemicals without incorporation of specific standards. Like IM-MS, the financial burden of the instrument, limits access to the instrument.

Third, GC-MS is widely accessible across scientific laboratories and is the instrument of choice for many regulatory and scientific protocols. However, it has the lowest resolution of the three instruments. As a result, its separation capacity is easily overwhelmed by the chemical complexity of petroleum substances.

Despite the range of advantages and disadvantages across the analytical instruments, no instrument outperformed the other in its ability to effectively group the petroleum substances, underscoring that molecular compositional analysis alone, lacks a comprehensive picture for these complex substances.[1,4,10] These findings reaffirm the challenges generated by UVCBs for manufacturers and regulatory institutions in regard to human health risk assessment.

Current regulatory requirements demand a robust range of human heath assessments, factoring heath effects, routes of exposure, and susceptible populations on a individual product or chemical basis.[1,2,3] Since the majority of UVCBs lack specific data in many of these areas. The time, money, and labor necessary to generate the required data reduces the ability to meet regulatory requirement deadlines.[3,4,10] However, within each challenge lies novel opportunity. Advancements in biological profiling, supplemented with “free” chemical characterization information can provide additional clarity toward substance pattern analysis. Additionally,

advancements in statistical modeling have the potential to clearly define the most distinguishable features of complex substances, reducing time, labor, and data required to build comprehensive substance categorization to fulfill regulatory read across requirments.[19] Lastly, further application of IVIVE modeling toward the development OEDs for complex substances can facilitate risk prioritization of UVCBs based on population exposure vulnerability, focusing time, labor, and money on categories of UVCBs exhibiting the most health related risk.[5,6]

5.2 Limitations

This study focused its design to pilot protein binding and molecular characterization techniques across four refinement categories of a previously studied sample set.[4,10] While increasing the tested sample size would provide a more complete pattern analysis, previous findings depicting efficient statistical grouping via biological response and heteroatom content, narrowed the sample set to one sample per refinement category.[4,10]

Second, only one analytical instrument, GC-MS, was used to characterize petroleum extract composition and evaluate cell medium protein binding. Additional laboratories have characterized the petroleum extracts using IM-MS. However, analytical evaluation of an instrument’s ability to categorize the extract samples was beyond the scope of this study.

Third, HFO samples were excluded from the instrument categorization comparison analysis due to no available GCxGC-FID data on the substances. Furthermore, instrument analytical methods used characterize neat petroleum substance were not uniform across all three instruments, in regard to molecular compounds analyzed. As a result, instruments were not evaluated on their ability to detect one compound over another, but rather on their holistic ability to effectively group the substances with respect to its refinement process.

Lastly, the hydrophobic nature of these petroleum samples highlights an additional complexity in regard to their utilization for in vitro assays. Albeit a fraction of a sample's aromatic content transfers to the DMSO extract, larger molecular weight ARCs (3 aromatic rings or more) remain above their water solubility limit through multiple dilutions. This can be alleviated by increasing the overall DMSO content in the in vitro assay, but at the detriment of cell viability. An alternative approach, using the water soluble fraction (WSF) of each petroleum sample would ensure all chemicals remain in solution.[27] However, this would reduce the ability to conduct dose response measurements, limiting the informational value for health assessments.

5.3 Conclusions

In summary, this study identified limitations in the utility of the RED device for protein binding evaluation of environmental chemicals. Additionally, this study established a suitable alternative method to evaluate hydrophobic environmental chemicals through the utilization of SPME techniques. This study subsequently demonstrated the ability of SPME to evaluate protein binding for complex substances. Through the characterization of petroleum extracts employed in biological profiling in vitro assays, this study illustrated that a compound's physicochemical properties, polarity and water solubility, influence the determination of the bio-available fraction generating abnormal cellular responses. Lastly, this study evaluated substance categorization efficiency across multiple analytical instruments, finding that no instrument significantly outperformed the others in categorizing complex substances. Consequently, this result reaffirms molecular class compositional analysis cannot be the only metric toward effective substance characterization. Together, this body of evidence codifies the challenges toward regulatory health assessment of complex substances. However, incorporating IVIVE modeling and clearly defining of the most distinguishable features of complex substances,

through statistical pattern analysis, may enable regulatory institutions to effectively prioritize UVCBs based on population exposure vulnerability, facilitating more robust human health risk assessment.

5.4 Future projects

Additional work related to this study will strengthen conclusions, regarding implementing OED evaluations to prioritize health risk assessment for complex substances. Implementation of in vitro hepatic clearance assays for the four petroleum substances, evaluated under cell medium protein binding, will obtain the data necessary to apply IVIVE modeling and OEDs. Evaluating the resulting OEDs against current exposure estimations can pilot a risk prioritization approached for the health evaluation of complex substances. Additionally, expanding the data pool, e.g., evaluating protein binding and metabolic clearance for additional petroleum substances will provide a more comprehensive data for IVIVE modeling. Lastly, information obtained from petroleum extract composition using additional instruments (IM-MS), protein binding, and hepatic clearance, creates an opportunity to conduct more detailed feature selection analysis toward complex substance characterization, augmenting biological profiling assay results to reduce regulatory concerns regarding its applicability toward regulatory read across.

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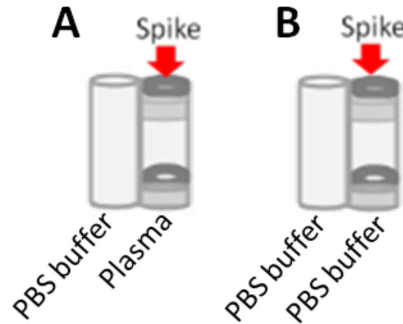

Figure 1. RED insert sampling and equilibrium control diagram.
RED insert plasma protein binding sampling method (A). Phosphate buffer saline (PBS) is applied into both chambers to evaluate chemical equilibration in the absence of protein (B).

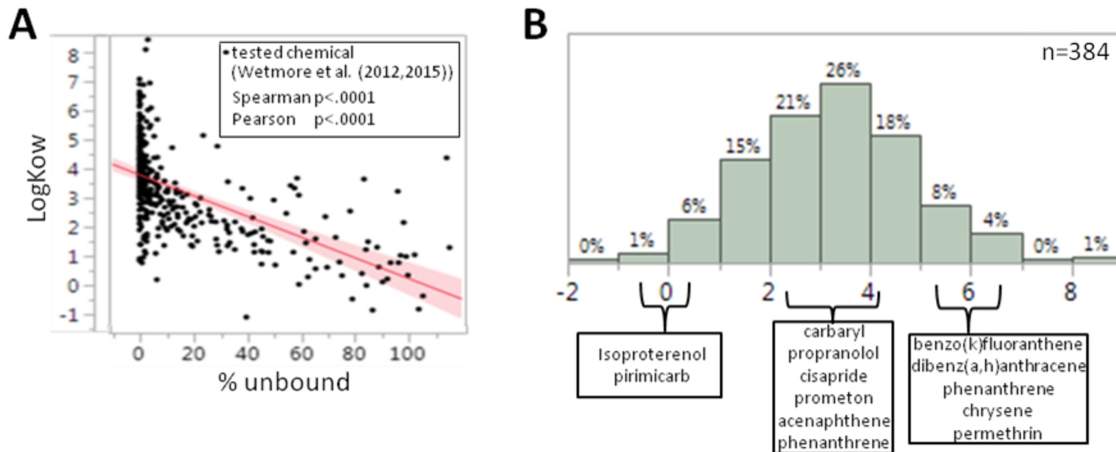

Figure 2. LogKow analysis of published RED results.
Correlation plot shows published RED unbound concentration values for 384 environmental chemicals (represented as dots) and their corresponding $LogK_{OW}$ values (A). Histogram depicting $LogK_{OW}$ distribution of previously tested 384 chemicals along with 13 chemicals (listed in brackets) selected in this study to evaluate the RED device (B).

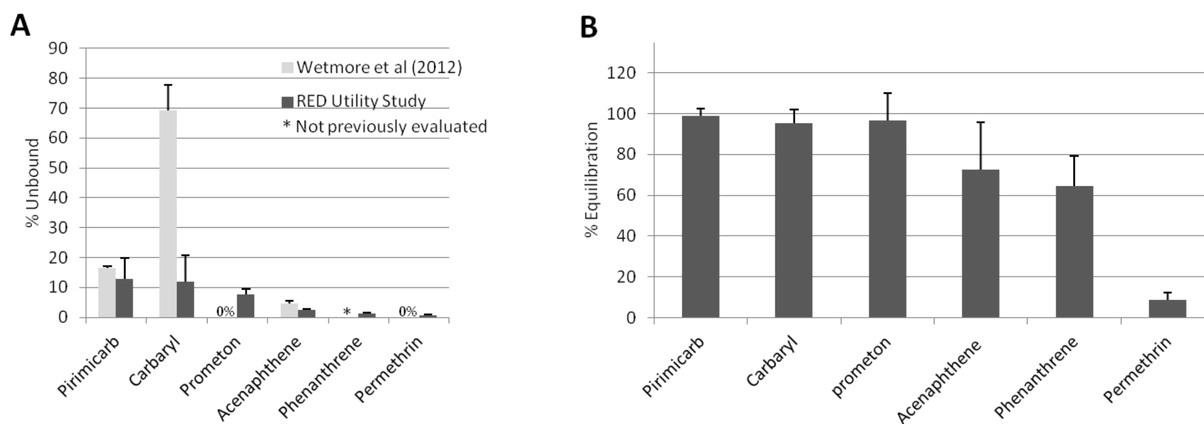

Figure 3. Equilibration controls evaluating published RED results for environment chemicals. Published RED plasma protein binding values across 6 environment chemicals (gray bars) vs this study (black bars) (A). These chemicals were subsequently evaluated on their ability to equilibrate within the RED device in the absence of protein. The bars reflect the concentration of chemical in each chamber after a 4 hour incubation period (B).

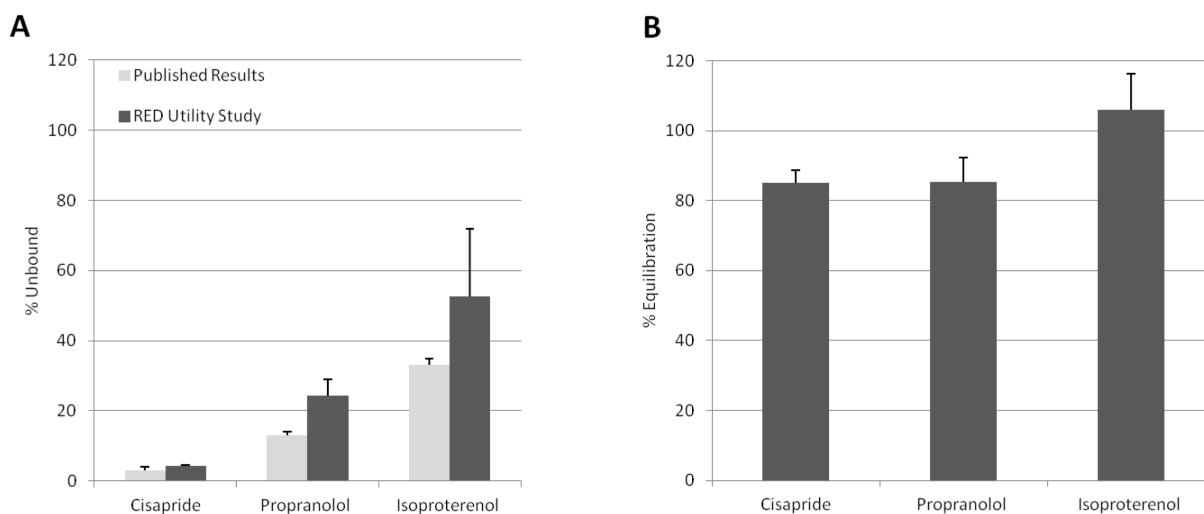

Figure 4. Equilibration controls evaluating published RED results for pharmaceuticals. Published RED plasma protein binding values across 3 pharmaceuticals (gray bars) vs this study (black bars) (A). All 3 pharmaceuticals retain a $LogK_{OW} < 5$. These chemicals were subsequently evaluated on their ability to equilibrate within the RED device in the absence of protein. The bars reflect the concentration of chemical in each chamber after a 4 hour incubation period (B).

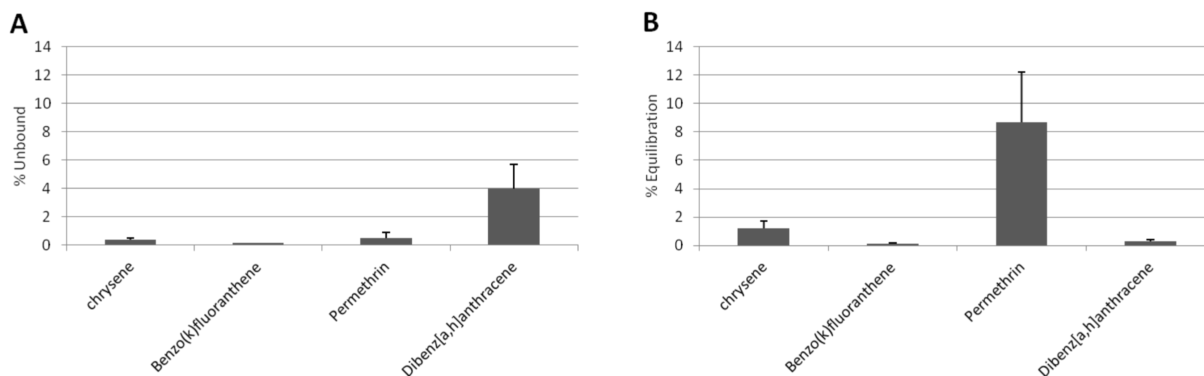

Figure 5. RED utility assessment via hydrophobic chemicals (LogK_{OW} >5).
The plot displays RED plasma protein binding results across 4 hydrophobic chemicals (A). These chemicals were subsequently evaluated by RED equilibration controls. The bars reflect the concentration of chemical in each chamber after a 4 hour incubation period (B).

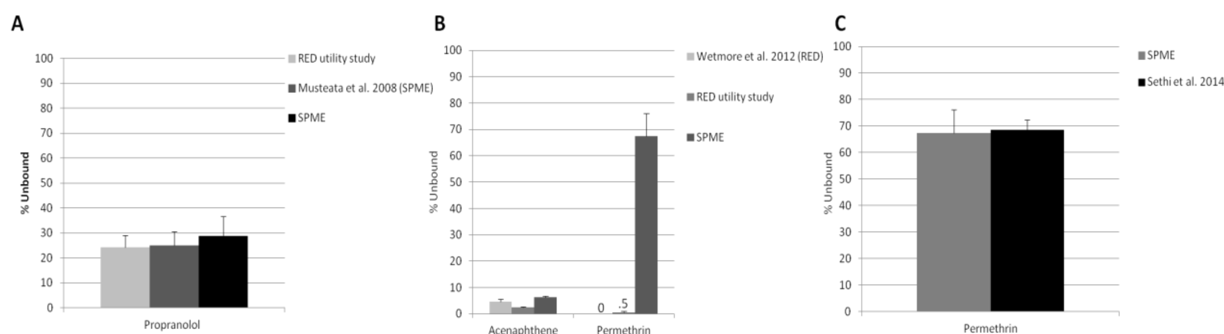

Figure 6. Plasma protein binding evaluation via SPME.
Left plot displays propranolol protein binding values across RED device (light gray), published SPME analysis (dark gray) and this study (black) (A). Middle plot shows plasma protein binding values for two hydrophobic chemicals using RED published data (light gray) and this study, binding using RED and SPME (B). SPME derived protein binding values for permethrin (gray) and compared to binding values using a published carbon labeled organic solvent extraction technique (C).

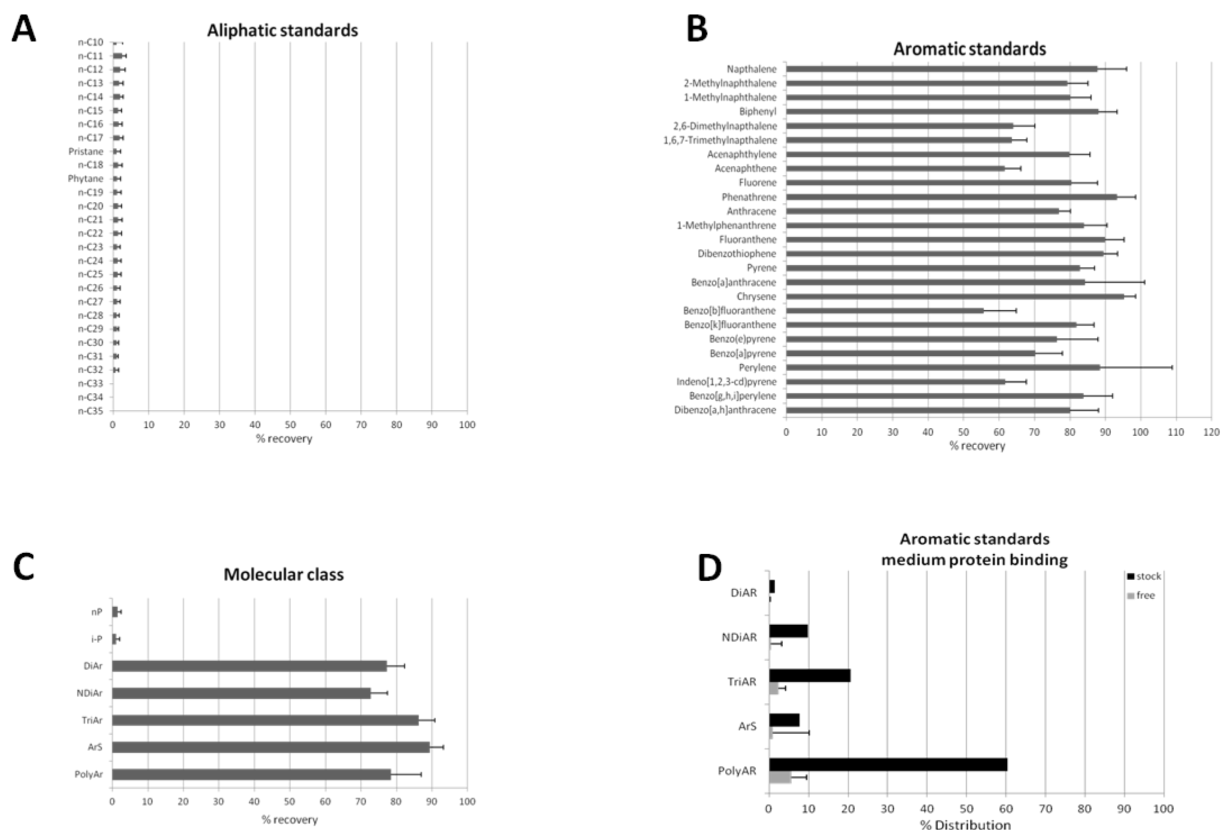

Figure 7. Characterization of petroleum DMSO extraction procedure using analytical standards. Upper left plot shows percent recovery of an aliphatic standard cyclohexane solution post DMSO extraction procedure (A). Upper right plot shows percent recovery of an aromatic standard cyclohexane solution post DMSO extraction procedure (B). Lower left plot displays DMSO extraction recovery by molecular class, aliphatic standards (nP and i-P) and aromatic standards (DiAr, NDiAr, TriAr, ArS, PolyAr) (C). Lower right plot displays cell medium protein binding of an aromatic standard mixture at .1nM concentration. Black bars represent percent concentration of aromatic standard stock mixture. Gray bars reflect unbound concentration post 4 hour incubation determined through SPME (D).

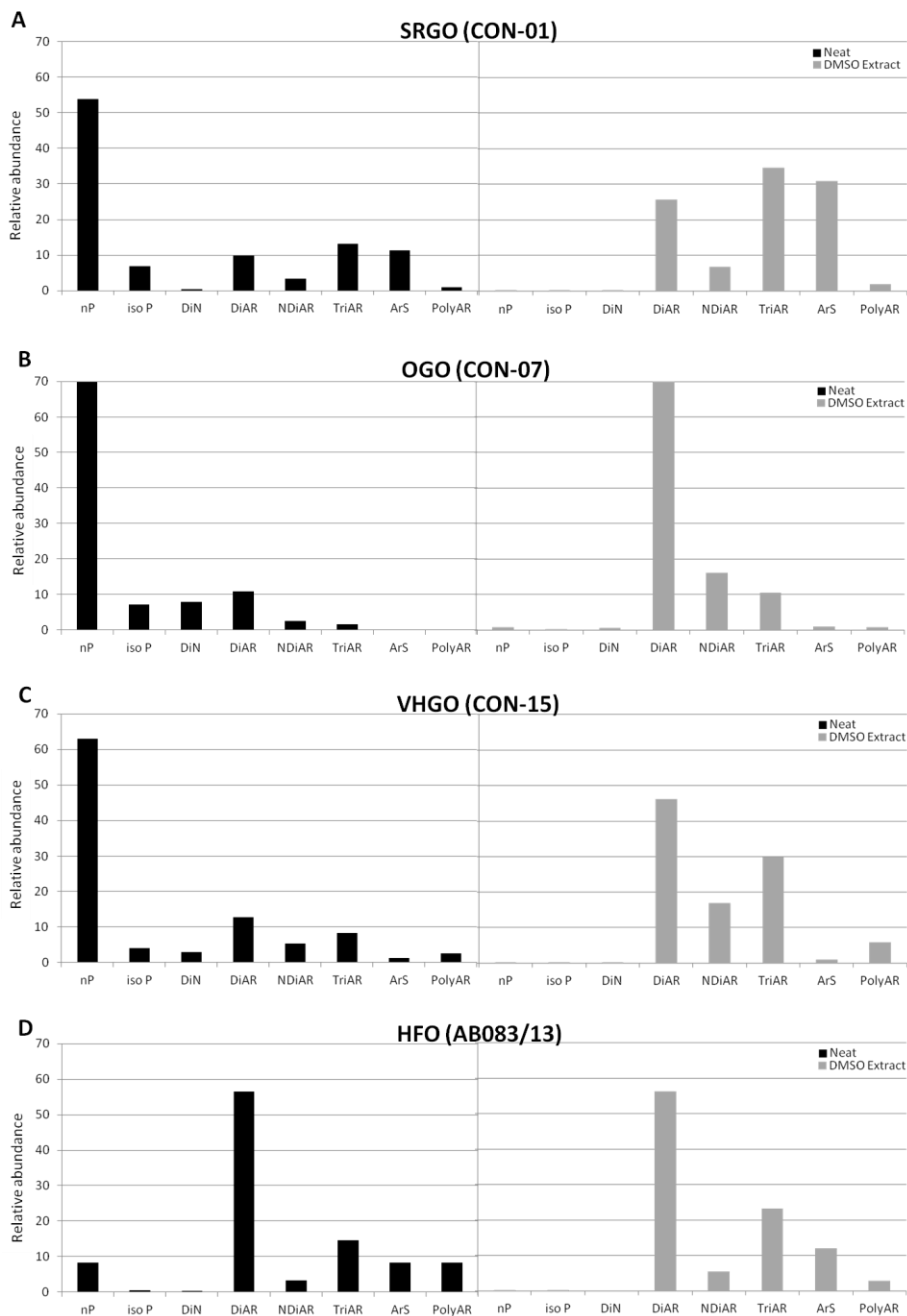

Figure 8. Petroleum neat vs extract molecular class compositional profile.
Black bars represent relative abundance values for eight chemical molecular classes across four separate neat petroleum substances. Light gray bars represent relative abundance values of the same molecular classes post DMSO extraction. Substances were analyzed via GC-MS.

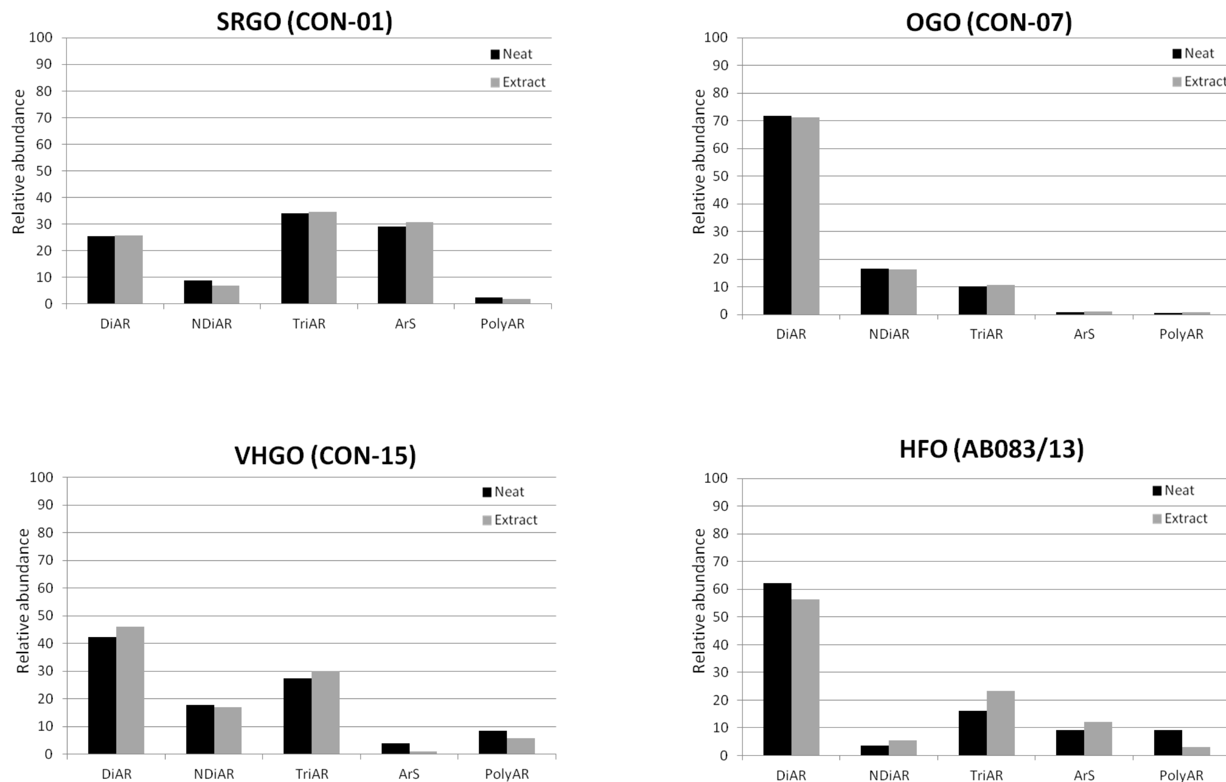

Figure 9. Neat vs extract aromatic relative abundance comparison.
Black bars display distribution of aromatic molecular classes within the neat petroleum substance. Gray bars display distribution of aromatic molecular classes within the DMSO extract. Relative abundance values were normalized to overall aromatic content in neat or extract substances.

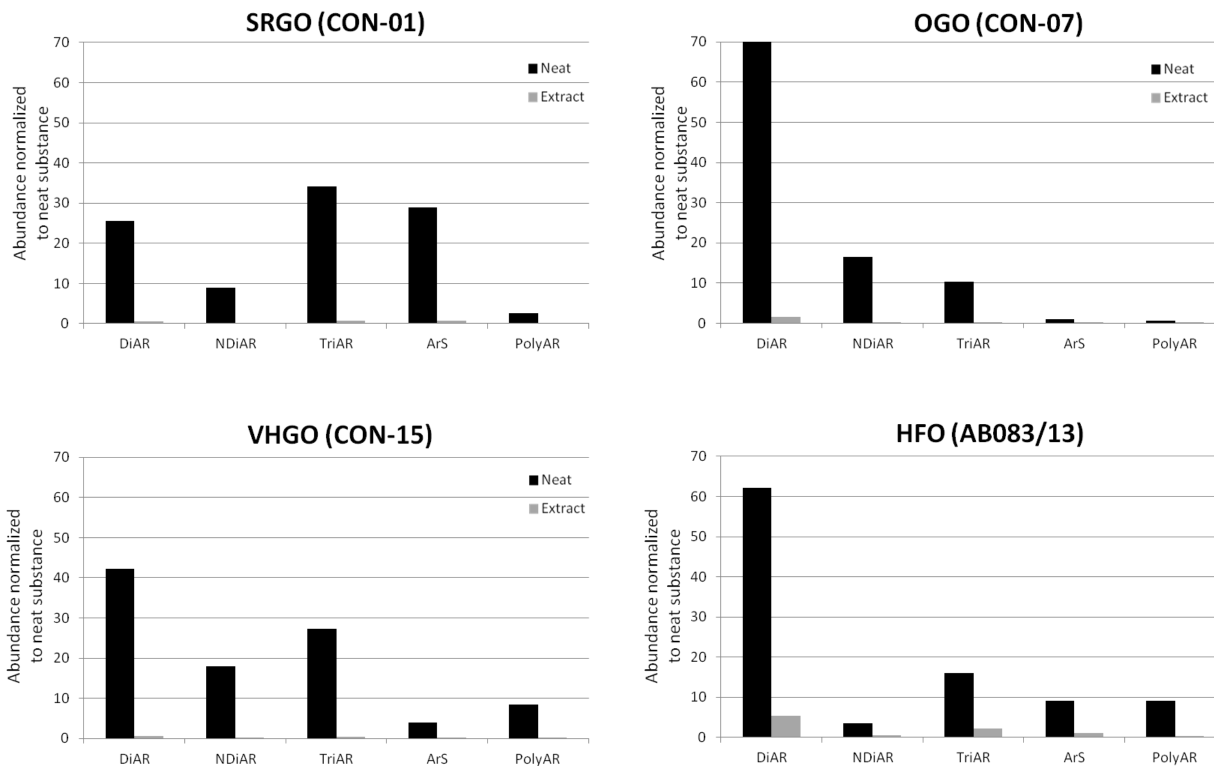

Figure 10. Extract total aromatic quantity vs neat petroleum substance.
Black bars display distribution of aromatic molecular classes within the neat petroleum substance. Gray bars show the fraction of aromatic content extracted with DMSO relative to the total amount present in the neat substance.

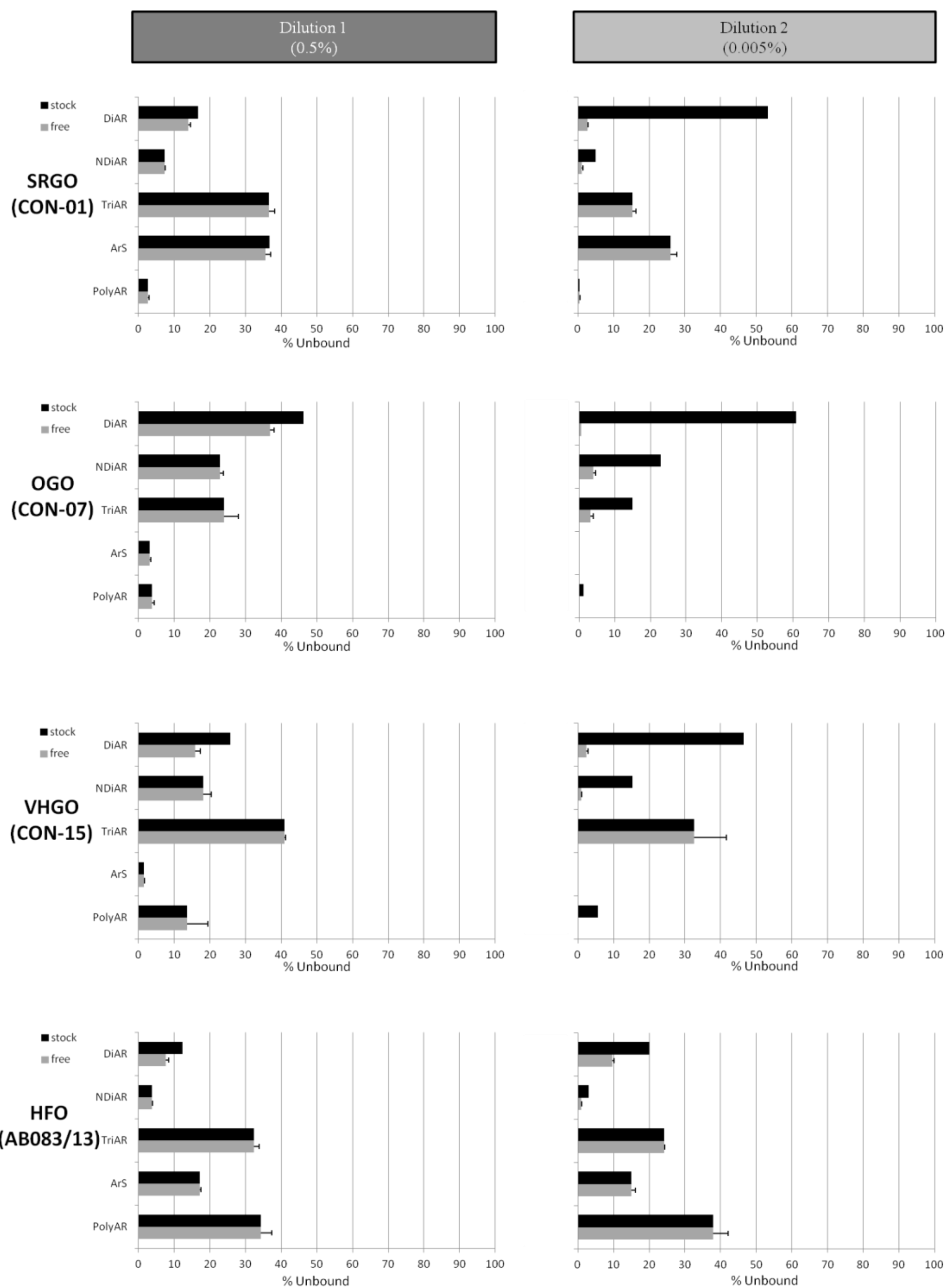

Figure 11. Aromatic unbound (free) concentration within petroleum extracts.
Free concentration (gray bars) is plotted relative to stock solution (black bars). Protein binding analysis was performed via SPME at two dilutions, 0.5% and 0.005% of the beginning DMSO extract.

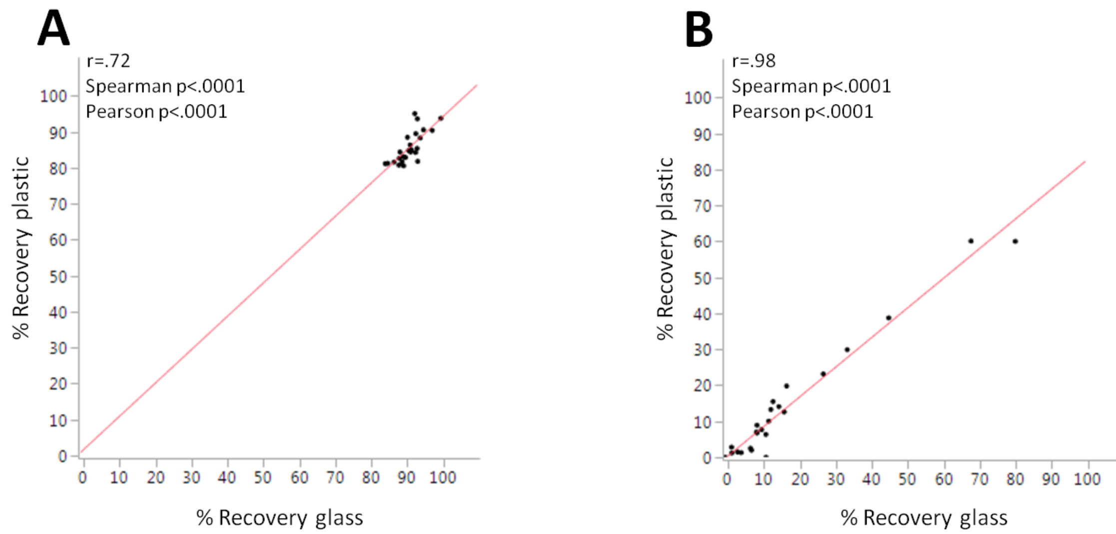

Figure 12. 384 well in vitro plate non-specific binding assessment via analytical standards. Recovery correlation plot of PAH analytical standards dissolved in DMSO solution applied to 384 well in vitro plates designs (plastic and glass) post 4 hour incubation (A). Recovery of the same PAH analytical standards dissolved in cell culture medium without proteins post 4 hour incubation (B).

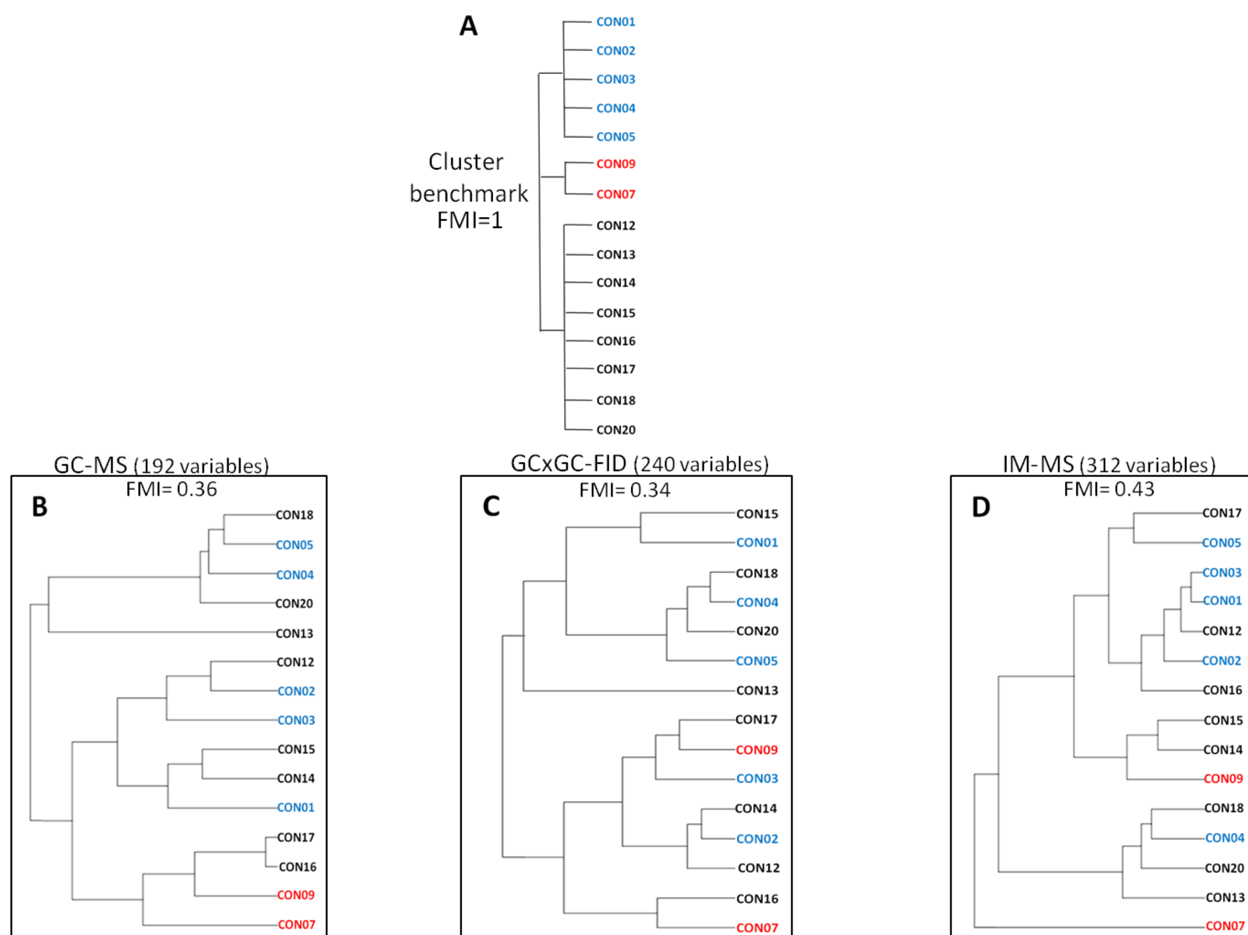

Figure 13. Hierarchical clustering of petroleum substances via separate analytical instruments. Hierarchical cluster dendrogram representing perfect grouping of 15 petroleum substance according to their refinement process, achieving a FMI=1 or perfect fit (A). Pearson correlation values were evaluated through chemical compositional analysis of each petroleum substance using three separate instruments. Potential correlation values were analyzed using average linkage hierarchical clustering (B,C, D).

APPENDIX B TABLES

Table 1.
RED assay utility evaluation chemical list

Chemical	CAS#	Type	$LogK_{OW}$
isoproterenol	51-30-9	pharmaceutical	.1
pirimicarb	23103-98-2	insecticide	1.7
carbaryl	63-25-2	insecticide	2.36
prometon	1610-18-0	herbicide	2.99
cisapride	260779-88-2	pharmaceutical	3.18
propranolol	318-98-9	pharmaceutical	3.48
acenaphthene	83-32-9	industrial	3.92
phenanthrene	85-01-8	industrial	4.46
chrysene	218-01-9	industrial	5.81
benzo(k)fluoranthene	207-08-9	industrial	6.11
permethrin	52645-53-1	insecticide	6.5
dibenz(a,h)anthracene	53-70-3	industrial	6.75

Table 2.

RED utility study instrumental parameters for chemical analysis by HPLC/MS

Chemical Name	CAS #	Mode	MRM[a]	Dw [b]	F[c]	CE[d]	CAV [e]
Carbaryl	63-25-2	+	202/145	200	100	13	4
Cisapride	260779-88-2	+	466/184	30	110	30	4
Isoproterenol	5984-95-2	+	212.1/194	30	82	9	4
			212.1/152*	30	82	17	4
			212.1/107*	30	82	33	4
Isoxaben	82558-50-7	+	332.7/165.2	200	130	23	4
Pirimicarb	23103-98-2	+	237/182	200	130	23	4
Propranolol	318-98-9	+	260/183	30	110	20	4
			260/116*	30	110	20	4
Sotalol	959-24-0	+	273/255	30	110	10	4
			273/213*	30	110	20	4

[a]MRM = MS/MS ion transitions (amu); [b]Dw = Dwell (msec); [c]F = Fragmentor (Volts); [d]CE = Collision Energy (Volts); and [e]CAV = Cell Accelerator voltage (Volts).

Additional MS parameters are as follows: Ion spray voltages were +3500 V for positive ion analysis; Gas temperature was set to 300^0C; Gas flow set to 10l/min; nebulizer set to 35psi; sheath gas temperature set to 350^0C with a gas flow of 11l/min; nozzle voltage set to 1000 V. Qualifier parameters for analytes marked with an *.

Table 3.
RED utility study instrumental parameters for chemical analysis by GC/MS

Chemical Name	CAS #	Ion 1	Ion 2	Retention Time (min)
Acenaphthene	83-32-9	154	153	10.515
Benzo(k)fluoranthene	207-08-9	252	253	28.412
Chrysene	218-01-9	228	226	25.668
Dibenz(a,h)anthracene	53-70-3	278	279	32.641
Permethrin (cis) isomer	52645-53-1	183	163	27.555
Permethrin (trans) isomer	52645-53-1	183	163	27.693
Phenanthrene	85-01-8	178	176	15.672
Prometon	1610-18-0	210	225	14.604
Internal Standards				
d10- Acenaphthene	15067-26-2	164	162	10.435
d12-Benzo(a)pyrene	63466-71-7	264	260	29.077
d12-Chrysene	1719-03-5	240	263	25.557
d10-Fluorene	81103-79-9	176	174	12.057
d8-Naphthalene	1146-65-2	136	134	6.563
d12-Perylene	152-96-3	264	260	29.288
d10-Phenanthrene	1517-22-2	188	184	15.587

Table 4.
Polycyclic-aromatic and saturated hydrocarbon standard listing

Chemical Name	CAS #	Chemical Name	CAS #
Polycyclic- aromatic hydrocarbons		saturated hydrocarbons	
Acenaphthene	83-32-9	n-C10	124-18-5
Acenaphthylene	208-96-8	n-C11	1120-21-4
Anthracene	120-12-7	n-C12	112-40-3
Benzo(e)pyrene	192-97-2	n-C13	629-50-5
Benzo[a]anthracene	56-55-3	n-C14	629-59-4
Benzo[a]pyrene	50-32-8	n-C15	629-62-9
Benzo[b]fluoranthene	205-99-2	n-C16	544-76-3
Benzo[g,h,i]perylene	191-24-2	n-C17	629-78-7
Benzo[k]fluoranthene	207-08-9	Pristane	1921-70-6
Biphenyl	92-52-4	n-C18	593-45-3
Chrysene	218-01-9	Phytane	638-36-8
Dibenzo[a,h]anthracene	53-70-3	n-C19	629-92-5
Dibenzothiophene	132-65-0	n-C20	112-95-8
Fluoranthene	206-44-0	n-C21	629-94-7
Fluorene	86-73-7	n-C22	629-97-0
Indeno[1,2,3-cd)pyrene	193-39-5	n-C23	638-67-5
Napthalene	91-20-3	n-C24	646-31-1
1-Methylnaphthalene	90-12-0	n-C25	629-99-2
2-Methylnaphthalene	91-57-6	n-C26	630-01-3
2,6-Dimethylnapthalene	581-42-0	n-C27	593-49-7

Table 4 (continued)

Chemical Name	CAS #	Chemical Name	CAS #
Polycyclic- aromatic hydrocarbons		saturated hydrocarbons	
1,6,7-Trimethylnapthalene	2245-38-7	n-C28	630-02-4
Perylene	198-55-0	n-C29	630-03-5
Phenathrene	85-01-8	n-C30	638-68-6
1-Methylphenanthrene	832-69-9	n-C31	630-04-6
Pyrene	129-00-0	n-C32	544-85-4
		n-C33	630-05-7
		n-C34	14167-59-0
		n-C35	630-07-9

Table 5.
Instrumental parameters for petroleum analysis by GC/MS

Chemical Name	Molecular class	Carbon number	Ion 1	Ion 2	Retention Time (min)	Cx interval
nC5	n-P	5	57		1.716	
nC6	n-P	6	57		1.979	
nC7	n-P	7	57		2.391	
nC8	n-P	8	57		2.529	
nC9	n-P	9	85		5.697	
nC10	n-P	10	85	113	8.086	
nC11	n-P	11	85	113	10.604	
nC12	n-P	12	85	113	13.069	
nC13	n-P	13	85	113	15.428	
nC14	n-P	14	85	113	17.658	
nC15	n-P	15	85	113	19.770	
nC16	n-P	16	85	113	21.762	
nC17	n-P	17	85	113	23.658	
Pristane	i-P	19	85	113	23.720	
nC18	n-P	18	85	113	25.456	
Phytane	i-P	20	85	113	25.566	
nC19	n-P	19	85	113	27.166	
nC20	n-P	20	85	113	28.804	
nC21	n-P	21	85	113	30.362	
nC22	n-P	22	85	113	31.862	
nC23	n-P	23	85	113	33.298	
nC24	n-P	24	85	113	34.673	
nC25	n-P	25	85	113	35.999	
nC26	n-P	26	85	113	37.273	
nC27	n-P	27	85	113	38.520	
nC28	n-P	28	85	113	39.825	
nC29	n-P	29	85	113	41.203	
nC30	n-P	30	85	113	42.645	
nC31	n-P	31	85	113	44.145	
nC32	n-P	32	85	113	45.712	
nC33	n-P	33	85	113	47.307	
nC34	n-P	34	85	113	48.969	
nC35	n-P	35	85	113	50.937	
2,3 dimethyl heptane	i-P	9	85			8-9
2244688 heptamethylnonane	i-P	16	85			13
2,6,10 trimethyldodecane	i-P	15	85			13-14
Decalin	DiN	10	138			10-11
C1- Decalins	DiN	11	152			11-12
C2- Decalins	DiN	12	166			11-13
C3- Decalins	DiN	13	180			13-14
C4- Decalins	DiN	14	194			13-15
Naphthalene	DiAr	10	128	127		11-12
C1- Naphthalenes	DiAr	11	142	141		13-14
C2- Naphthalenes	DiAr	12	156	141		14-15
C3- Naphthalenes	DiAr	13	170	155		15-17
C4- Naphthalenes	DiAr	14	184	169,141		15-18

Table 5 (continued)

Chemical Name	Molecular class	Carbon number	Ion 1	Ion 2	Retention Time (min)	Cx interval
Biphenyl	DiAr	12	154	153		14
Acenapthylene	NDiAr	12	152	153		14-15
Acenaphthene	NDiAr	12	154	153		15-16
Dibenzofuran	NDiAr	12	168	169		15-16
Fluorene	NDiAr	13	166	165		16-17
C1- Fluorenes	NDiAr	14	180	165		17-18
C2- Fluorenes	NDiAr	15	194	179		18-19
C3- Fluorenes	NDiAr	16	208	193		19-21
Phenanthrene	TriAr	14	178	176		18-19
Anthracene	TriAr	14	178	176		18-19
C1- Phenanthrenes	TriAr	15	192	191		19-20
C2- Phenanthrenes	TriAr	16	206	191		20-23
C3- Phenanthrenes	TriAr	17	220	205		21-23
C4- Phenanthrenes	TriAr	18	234	219,191		22-25
Dibenzothiophene	ArS	12	184	152,139		17-18
C1- Dibenzothiophenes	ArS	13	198	184,197		18-20
C2- Dibenzothiophenes	ArS	14	212	197		19-21
C3- Dibenzothiophenes	ArS	15	226	211		20-23
C4- Dibenzothiophenes	ArS	16	240			21-24
Fluoranthene	PolyAr	16	202	101		21
Pyrene	PolyAr	16	202	101		21-22
C1- Fluoranthenes/ Pyrenes	PolyAr	17	216	215		22-24
C2- Fluoranthenes/ Pyrenes	PolyAr	18	230	215		23-25
C3- Fluoranthenes/ Pyrenes	PolyAr	19	244	229,215		25-27
Benz(a)anthracene	PolyAr	18	228	226		25-26
Chrysene	PolyAr	18	228	226		25-26
C1- Chrysenes	PolyAr	19	242	241		26-27
C2- Chrysenes	PolyAr	20	256	241		27-29
C3- Chrysenes	PolyAr	21	270	255		28-30
C4- Chrysenes	PolyAr	22	284	269,241		29-32
Benzo(b)fluoranthene	PolyAr	20	252	253,125		28-29
Benzo(k)fluoranthene	PolyAr	20	252	253,125		28-29
Benzo(e)pyrene	PolyAr	20	252	253		29-30
Benzo(a)pyrene	PolyAr	20	252	253,125		29-30
Perylene	PolyAr	20	252	253		29-31
Indeno(1,2,3-c,d)pyrene	PolyAr	22	276	277,138		32-33
Dibenz(a,h)anthracene	PolyAr	22	278	279,139		32-33
Benzo(g,h,i)perylene	PolyAr	22	276	277,138		32-34

Table 6.

Example of petroleum two dimensional analytical matrix.

Petroleum substance OGO CON-07 GC-MS

Carbon Number	Molecular Class								
	nP	iso P	DiN	ArS	DiAR	NDiAR	TriAR	PolyAR	Total
5	0								0
6	2.734006								2.734006
7	0.021359								0.021359
8	0								0
9	1.249564	0.292009							1.541573
10	1.34518		0.387617		0.317769				2.050565
11	2.129025		1.348667		1.477748				4.95544
12	5.012709		2.611793	0	3.982412	0.863592			12.47051
13	7.923805		2.888001	0.139384	3.311475	0.176824			14.43949
14	8.766838		0.612054	0	1.757384	0.372852	0.13333		11.64246
15	8.887513	1.899569		0		0.622265	0.393548		11.8029
16	7.718343	0		0		0.464432	0.656538	0.088861	8.928175
17	6.776078						0.317426	0	7.093504
18	5.41672						0.050522	0.007142	5.474384
19	4.159762	2.648289						0	6.808051
20	3.150595	2.386677						0	5.537273
21	2.140363							0	2.140363
22	1.197351							0	1.197351
23	0.636931								0.636931
24	0.283123								0.283123
25	0.131655								0.131655
26	0.067137								0.067137
27	0.030313								0.030313
28	0.013447								0.013447
29	0								0
30	0								0
31	0								0
32	0								0
33	0								0
>33	0								0
									100
Total	69.79182	7.226544	7.848132	0.139384	10.84679	2.499966	1.551365	0.096004	100

Table 6 (continued)

Petroleum substance OGO CON-07 GCxGC-FID

Carbon Number	Molecular Class									
	nP	isoP	N	DiN	MonoAr	NMonoAr	DiAr	NDiAr	TriAr	Total
5	0.00	0.00	0.00							0.01
6	0.01	0.00	0.02	0.00	0.00					0.03
7	0.01	0.01	0.08	0.00	0.02					0.13
8	0.04	0.04	0.18	0.01	0.10					0.36
9	0.11	0.10	0.30	0.06	0.32	0.01				0.89
10	0.30	0.29	0.54	0.29	0.51	0.16	0.01			2.10
11	0.56	0.49	0.80	0.78	0.59	0.74	0.10			4.05
12	0.84	0.66	1.25	1.03	0.71	1.27	0.32			6.09
13	1.17	1.16	1.58	1.43	0.83	1.47	0.51	0.04		8.19
14	1.46	1.51	2.02	1.50	0.84	1.48	0.64	0.13	0.00	9.58
15	1.67	1.70	2.12	1.06	0.98	1.23	0.50	0.25	0.04	9.54
16	1.65	1.93	2.12	0.82	0.91	0.98	0.39	0.32	0.06	9.18
17	1.64	1.57	2.48	0.85	0.85	0.88	0.39	0.13	0.07	8.85
18	1.55	1.92	2.14	0.52	0.74	0.67	0.36	0.13	0.03	8.06
19	1.43	2.37	2.15	0.71	0.76	0.73	0.17	0.04	0.00	8.36
20	1.36	2.05	1.89	0.53	0.59	0.41	0.14	0.03	0.00	6.99
21	1.18	1.33	1.60	0.41	0.46	0.31	0.11	0.01	0.00	5.41
22	0.99	1.06	1.51	0.17	0.37	0.22	0.09	0.01	0.00	4.41
23	0.76	0.96	1.06	0.12	0.17	0.10	0.02	0.00	0.00	3.19
24	0.50	0.61	0.61	0.10	0.09	0.07	0.02	0.00	0.00	1.99
25	0.31	0.52	0.28	0.04	0.06	0.04	0.00	0.00	0.00	1.25
26	0.15	0.38	0.23	0.04	0.02	0.02	0.00	0.00	0.00	0.85
27	0.07	0.12	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.22
28	0.03	0.08	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.14
29	0.01	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.06
30	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
31	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
>33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
										100.00
Total	17.81	20.93	25.04	10.45	9.92	10.80	3.76	1.09	0.20	100.00

Table 6 (continued)

Petroleum substance OGO CON-07 IM-MS

Carbon Number	Molecular Class								
	MonoAr	NMAr	DiAr	NDiAr	TriAr	ArS	PolyAr	PolyN	Total
5									
6									
7									
8									
9		0.1014							0.101448
10		0.0833	0.2416						0.324925
11		0.0515	0.2503						0.301838
12	0.0093	0.0313	0.4203	0.8931		0.0623	0.2468		1.663075
13		0.0248	2.4664	1.0249		0.0044			3.520395
14	0.0100	0.3286	3.4074	3.2008					6.946723
15	0.0106	0.2156	1.4074			0.0048	7.4681		9.106525
16		0.1387	1.0283	8.2718		0.0231	3.2320		12.69392
17	0.0145	0.1036	0.6211	11.3593		0.1054	1.8880		14.0919
18	0.0155	0.1614	0.4536	11.7176	1.6868		0.0670		14.10195
19		0.0097	0.3625	8.7651			3.1967		12.3339
20		0.0850	0.2631	6.0582			2.7555	0.0039	9.165674
21		0.2061	1.2644	2.5348			1.5161	0.0045	5.525826
22		0.0350	0.7347	1.4702			1.4582	0.0031	3.701234
23		0.3959		0.7486			1.1905	0.0033	2.338295
24		0.0138	0.2960	0.4374			0.2978		1.045021
25			0.1459	0.2452			0.6585		1.049591
26			0.1104				0.5569		0.667316
27	0.1121		0.0525	0.0999			0.2689		0.533329
28			0.0371	0.0058			0.0722	0.0045	0.119511
29			0.0125				0.2469		0.259482
30		0.0358	0.0366	0.0306			0.2837		0.386598
31									0
32		0.0215							0.021518
33									0
>33									0
									100
Total	0.171968	2.043056	13.61198	56.86347	1.686777	0.199925	25.40352	0.019304	100