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Influence of Metal-Working Fluid Formulations on Dermal Absorption of Biocides

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LIST OF ABBREVIATIONS

MWF	Metal-working fluids
MCF	Membrane-coated fibers
PDMS	Polydimethyl siloxane
PA	Polyacrylate
CW	Carbowax
PANI	Polyaniline
ANOVA	Analysis of variance
Log $K_{O/W}$	Log octanol/water partition coefficient
Log $K_{pdms/w}$	Log PDMS/water partition coefficient
MO	Mineral oil
PEG	Propylene glycol 200
SC	Stratum corneum
SLS	Sodium lauryl sulfate
SO	Soluble oil
SS	Semi-synthetic MWF
SYN	Synthetic MWF
Kp	Skin permeability
Log $K_{(Skin/Astrocut)}$	Log skin permeability in Astrocut formulation

ABSTRACT: (500 WORDS)

When the skin of workers is exposed to different industrial formulations, it is often assumed that dermal absorption of harmful substances will vary according to the chemical composition of these different formulations. It is often assumed that this may explain why one formulation may be more harmful locally and/or systemically than another formulation. ***This project attempted to accurately predict these formulation effects at the chemical manufacturing end or onsite production end and thus provide useful quantitative information that will help mitigate occupational exposure to formulations that may promote dermal absorption of a potential toxicant***. The EPA Guidance Document on Dermal Risk Assessment (EPA, 2004) recommends a regression model for single chemicals but provides no guidance on mixtures or formulations. Our project developed regression models that was predictive of dermal absorption in various classes of industrial metal working fluids (MWFs). We then attempted to validate these trends in vivo with variable success.

The primary objective of this research was to *quantify chemical mixture interactions that are extrapolatable from solvatochromatic parameters associated with linear solvation energy relationships (LSER).* LSER is a powerful approach to assess the similarity and differences in the intermolecular forces associated with interactions between multi-phasic systems (e.g., skin and MWF formulation), and can by extension be used to examine intermolecular forces or chemical mechanisms associated with transdermal diffusion of solutes in a chemical mixtures environment. This LSER approach allows for quantifying and comparing mixture induced changes in a viable skin membrane and our MCF approach for screening. The membrane coated fiber (MCF) approach was limited to one MCF, PDMS fiber which closely reflects skin than the other MCFs. This approach was able to identify significant MWF formulation effects and concentration effects for limited number of solutes; the former was anticipated but we did not anticipate the latter. Using a series of flow-through diffusion experiments, we were able to develop for the first time a robust QSAR model for predicting skin absorption in the three most widely used MWFs. We were also able to validate to a limited extent some of these findings in an in vivo pig model. The information from these series of experiments should provide MWF manufacturer and workers who use these MWF, which formulations are more likely to increase skin absorption and with some knowledge of the physicochemical properties of the various MWF additives, which additives such as biocides are more likely to penetrate human skin.

SIGNIFICANT FINDINGS

Workers in the metal fabrication industry are more often exposed to metal working fluids (MWF) and its components such as biocides via the skin that can cause harm to the skin and/or the entire body if absorbed by the dermal route. Many of these workers are exposed to more than one chemical additive in any given MWF formulation, and there is little or no means of estimating what class of MWF formulations can result in increased or decreased absorption of biocides across skin. The work from this project developed a novel technique that models biocide absorption in skin on the basis of quantitative changes in physicochemical properties associated with the formulation interacting with a statistical regression model that was validated in skin *in vitro* and then *in vivo* to determine validity

of these models in an occupational dermal exposure. The following were the significant findings from this project:

- We extended the one-factor expanded Abraham model that now accounts for complex experimental conditions but also improve the ability to conduct the rigorous hypothesis testing. The analysis demonstrated that the expanded Abraham model not only has a better model fit and predictive power, but also the power of testing various scientific hypothesis. This analysis reveals that the partition theory is valid only under certain assumptions.
- A principal component analysis showed that the selected training set was representative of the chemical space relevant to chemical relevant to occupational exposure in the metalworking industry.
- Our QSAR work with in vitro skin demonstrated that skin permeability was shown to have the rank order for the following formulations relevant to the MWF: water > synthetic > semisynthetic > soluble oil. Our expanded QSAR model improved both the model fit and the predictive power when compared to the simple model in the previous grant period. More importantly, our data analysis implies that skin permeability in water or synthetic MWF is likely to be three (3) times greater than the permeability in soluble oil formulations. Our coefficients in our linear regression model accurately estimated permeability if there were changes in physicochemical parameters; for example, one unit increase in overall hydrogen bond basicity for a chemical additive in soluble oil will increase permeability by a factor of 117.5 provided all other chemical descriptors are fixed.
- This research was the first to demonstrate the dermal absorption of dicyclohexylamine (DCHA) and other related amines and the effects of MWF formulations on their skin absorption. DCHA is important as some in the industry use this amine amongst others as a biocide although it is registered only as a corrosive inhibitor in spite of it also having biocidal activities. We reported that hydrophilic amines are more likely to be absorbed in soluble oil formulations and that lipophilic amines such as DCHA were more likely to be absorbed in synthetic MWF formulations
- The in vivo transdermal studies were the first ever studies to assess biocide absorption across several (that is, four) MWF formulations. Our studies demonstrated skin absorption of the biocide, OPP, was similarly influenced by the MWF formulations as observed in the in vitro studies. Although there were several exceptions that were not predicted from the in vitro studies, the general trend was comparable. That is, the more aqueous the MWF such as the synthetic MWFs, the more likely that performance additives similar in chemistry to the OPP biocide, will be absorbed across skin and therefore be focus of future risk assessments.

TRANSLATION OF FINDINGS.

Contact with metalworking fluids containing biocides sometimes will cause skin irritation or even more harmful consequence. Thus, it is of interest to study the permeation capability of the added chemical solutes through skin and find safer chemical solutes that can be used as biocides or performance additives in metalworking fluids.

One of our first studies used the membrane-coated fiber (MCF) approach to assess physicochemical interactions for at least three formulations relevant to the metalworking

industry. The MCF approach was able to determine formulation effects on partitioning of chemicals in MWFs and determined that the concentration of these MWF can influence this partitioning behavior. The next series of studies reported the first algorithm (Baynes Rule) for selecting solutes to conduct a dermal absorption QSARs for MWFs and the other study developed an expanded QSAR model for dermal absorption of industrial metalworking fluid formulations with internal and external validation.

Our laboratory has identified MWF formulation effects in two classes of performance amines that will inform the risk assessment of these amines in industrial metalworking fluid formulations used in the working environment. Many of these amines are used as performance additives in MWF formulations, but we were able to determine that different formulations can either enhance or reduce skin absorption.

In conclusion, this project utilized several in vitro skin absorption methods to identify formulations effects on a large number of chemicals that can be related to those chemicals found in MWF and could result in occupational exposure. We are the first group to develop a regression model (quantitative structure active relationship, QSAR) that we have tested and validated to predict skin permeability for three of the most widely used generic MWFs used in industry. Our in vivo pig study validated for the most part the trends we observed in vitro. It should noted that this project focused on skin absorption parameters as we attempted to model and assess formulation effects and therefore predict the likely scenario for absorption in workers. However, not all chemicals need to be absorbed across the skin to illicit local irritant effects, and therefore the formulation effects we have dissected out in this project may not translate into comparable adverse health effects in works. Nevertheless, our models do provide guidance for future studies in this regard.

SCIENTIFIC REPORT

BACKGROUND FOR THE PROJECT:

(i) Dermal Absorption Assessment

Use of live animal models is prohibitively expensive and alone do not provide significant physicochemical information from which solute and solute mixture absorption can be extrapolated. Skin from humans as well as other laboratory animals is used in *in vitro* diffusion studies to obtain permeability data. Porcine skin is similar anatomically, physiologically, and biochemically to human skin (Monteiro-Riviere, 1991; 1996), it is more readily available, and many studies have demonstrated that permeation in porcine skin is comparable to human skin (Wester *et al*., 1998). Irrespective of the animal model used in dermal absorption research, the solute has to ***first*** diffuse across the outermost layer of the skin, the stratum corneum, which consists primarily of protein-rich corneocytes embedded in a lipid matrix.

Solutes such as MWF biocides must then traverse the more aqueous viable epidermis and then diffuse into the blood stream via the microvasculature at the epidermis-dermis juncture. However, it must be stressed that the stratum corneum layer is the rate-limiting barrier for absorption, and it is the ***intermolecular interactions*** between diffusing solutes and lipid components of the stratum corneum which dictate its final disposition in skin and absorption into the blood stream. This proposal is focused on using an inert

membrane model (MCF) to probe these interactions in the presence of MWF formulations that could significantly impact dermal risk assessment of industrial biocides.

Permeability is the preferred metric for extrapolating across dose in dermal risk assessment and also better suited for assessing and ultimately extrapolating across formulation effects which is the purpose of this grant application. Assuming that solutes obey Fick's first law of diffusion as they diffuse across the human epidermal membrane, the dermal permeability can be defined by the equation,

Permeability (cm/hr), Kp = J_{ss}/C_v ***Equation 1***

where J_{ss} represents the solute steady state flux and C_v the solute dosing concentration. Solute permeability is dependent on solute diffusivity, **D**, (cm^2/hr) in the membrane and its ability to partition from the dosing solution to the stratum corneum layer of skin. The latter is referred to as the stratum corneum-vehicle partition coefficient, $\mathbf{K_{sv}}$, and is often correlated with octanol-water partition coefficients, $K_{o/w}$. Permeability can therefore be re-defined by the equation,

$$\mathbf{Kp = \frac{D * K_{s/v}}{l}} \qquad \textit{Equation 2}$$

where ***l*** = membrane thickness. It is quite conceivable that when workers are exposed to a toxicant in the presence of a MWF formulation, that not only will the formulation alter the biological membrane, it will also influence the physicochemical interaction between the toxicant and skin. This influence will be reflected in changes in the partitioning behavior. This is ignored in current dermal human health risk assessments.

(ii) Quantitative Structure Permeability Relationship (QSPR) Models.

QSPR models have been used to relate physicochemical parameters to dermal permeability, but they have limitations primarily due to inadequate statistical fit and/or not being directly applicable to chemical mixtures or formulations. Many of the more recent QSPR models have been based on permeability (Kp) data compiled by Flynn (1990) for 94 compounds from numerous sources and experimental protocols that can however be described as being more heterogeneous than other chemical clusters or series previously analyzed for QSPR. This data was utilized to generate the now widely cited **Potts and Guy (1992) model** (**Equation 3**) that however reported a **poor fit** ($r^2 = 0.67$).

Log Kp = 0.71 log Ko/w – 0.0061MW – 6.3 ***Equation 3***

In spite of this poor fit, the US EPA has refined this model by excluding several experimental data points, and they have recommended that this refined model be utilized in predicting permeability (Kp) values. It is based on small hydrocarbons and pharmaceutical drugs that ***bear little resemblance to chemicals that may be of occupational concern***. Similarly, pharmaceutical formulations are designed to optimize penetration and bear no resemblance to occupational exposure conditions.

The more recent QSPR approaches now utilize such physicochemical descriptors as hydrophobicity (e.g., log Ko/w) as well as electronic properties (e.g., H-bonding), and steric properties (e.g., MW, MV) that are really ***solvation energy descriptors***. This has improved model predictions and will be our focus.

While **Equation 3** establishes a weak but plausible link between permeability and molecular partitioning and distribution between an organic/lipid phase and an aqueous phase, the solute partitioning behavior can be better described at the molecular level in terms of the free energy of solute transport. The latter is a function of solute size, polarity, and hydrogen bonding ability, and it is this ***solvatochromatic approach*** which has been used to determine a variety of physicochemical solute properties such as solubility (Kamlet *et al*., 1986; Abraham and Le, 1999), partition coefficients (Kamlet *et al*., 1988), and more recently bio-membrane permeability (Abraham *et al*., 1994; 1995). In this approach, a small set of solvatochromatic descriptors were combined in a linear fashion to correlate solute properties which results in a ***linear solvation energy relationship (LSER***). Any given LSER is basically a model of solute solvation in various solvent phases (solute-solvent interactions), and can by extension model solute-membrane interactions. It should be noted that the role of hydrogen bonding plays a significant role in the LSER, and has thus receives the most attention in QSPR modeling. A more recent Potts and Guy (1995) model (**Equation 4**) clearly demonstrated its importance as reflected in an improved fit (r^2 = 0.94) even in the absence of the hydrophobic descriptor, log Ko/w, but the inclusion of a different steric descriptor, molecular volume (MV).

$$\textbf{Log Kp} = \mathbf{0.0256\ MV - 1.72\ \Sigma\alpha_2^H - 3.93\ \Sigma\beta_2^H - 4.85} \qquad \textit{Equation 4}$$

The descriptors $\Sigma\alpha_2^H$ and $\Sigma\beta_2^H$ reflect solute hydrogen donor capacity and solute hydrogen acceptor bond capacity, respectively, and they measure the exoergic effects. In essence, these two descriptors can measure the ability to donate or accept a proton in a solute-solvent hydrogen bond. This model also provides basic ***physicochemical insight*** into epidermal permeability as hydrogen bonding activity is inversely related to permeability, and where hydrogen bond donor activity ($\Sigma\alpha_2^H$) can be more important than hydrogen bond acceptor activity ($\Sigma\beta_2^H$). This QSPR work was then followed-up by Abraham *et al*. (1999) and others, and as can be gleaned from **Equation 5** below, this model was not only an excellent fit (r^2 = 0.96) for a more diverse set of the Flynn (1990) solutes, but now includes other descriptors such as: molar fraction (R_2), which can be obtained from refractive index of solutes that are liquid at 20ºC; solute dipolarity/polarisability (π_2^H) which measures endoergic effects of solute-solvent dipole-dipole and dipole-induced dipole interactions; and McGowan characteristic molecular volume, V_x, which can be calculated from bond and atom contributions.

As proposed by more recent investigators (Trone and Khaledi, 2000; Abraham *et al.* 1999; Abraham and Martin, 2004), the above 5 independent molecular descriptors can be related in general terms to a given solute transport property (SP) such as $K_{o/w}$ or epidermal permeability as in **Equation 5** .

$$\textbf{Log SP} = \mathbf{c} + \boldsymbol{r} \bullet \mathbf{R_2} + \boldsymbol{s} \bullet \pi_2^H + \boldsymbol{a} \bullet \Sigma\alpha_2^H + \boldsymbol{b} \bullet \Sigma\beta_2^H + \boldsymbol{v} \bullet \mathbf{V_x} \qquad \textit{Equation 5}$$

This relationship (LSER) will be utilized in this proposed research to assess several physicochemical processes; namely, solute or biocide partitioning between a MWF formulation and an inert or biological membrane. This well tested LSER therefore provides a tool with which to understand chemical mechanism(s) dictating the physicochemical interactions when a membrane is exposed to a complex chemical formulation.

How is all of this related to the skin*?* The physicochemical nature of the stratum corneum (SC) and the effect of MWF formulation additives such as solvents or surfactants on its inherent physicochemical properties or membrane environment (Pugh *et al*., 1996) will influence membrane diffusivity and thus permeability. The SC has been reported to be a predominantly H-bonding donor **(α_2^H)** rather than acceptor (β_2^H) with an α_2^H/β_2^H ratio of 0.6/0.4 (Pugh *et al*., 1996). Intuitively, the presence of MWF additives can influence this ratio and thus enhance or retard solute diffusion across a membrane. Impregnation of a PDMS membrane (an inert barrier) with octanol to mimic the hydrogen bonding environment of the SC demonstrated that this solvent and not toluene (which has no H-bonding properties) significantly reduced the flux of various acids and alcohols. (Du Plessis *et al*., 2002). This observation strongly suggests that hydrogen bonding interaction was responsible for altered permeability and is thus an integral solvatochromatic parameter in this proposed application.

It must be emphasized that the 5 molecular descriptors described thus far are a small set of independent variables that have been combined in a linear fashion to correlate solute properties. This allows for a quantitative and qualitative identification of ***solute-mixture interactions*** that we propose to demonstrate in this application. In the initial experiments of this grant proposal, a training set of selected solutes with known molecular descriptors will be used to calibrate the membrane-coated MCF) fiber/water system. This MCF/water system will be used to experimentally determine the SP parameters in **Equation 5.**

The ***system/strength coefficients*** in equation 5 (***r, s, a, b, v***) represent the unique system coefficients for each system, and they can be obtained from multiple linear regression analysis. ***These system coefficients refer to the interaction properties of the solute with the solvent phase, and if there is more than one phase as is the case with skin, it encodes the difference in interaction properties of the two phases. These two phases can be Octanol+Water or can be MCF+Water, MCF+MWF, Skin+Water, or Skin+ MWF. With the exception of the former, the 4 latter systems are the primary systems that will evaluated in this grant application***. These important system or strength coefficients are thought to characterize the following physicochemical and biochemical properties of the system as described below:

a-strength coefficient: the phase hydrogen-bond acceptor strength,
b- strength coefficient: the phase hydrogen-bond donor strength;
s- strength coefficient: the phase or solution polarizability/dipolarity;
v- strength coefficient: the phase hydrophobicity.
r- strength coefficient: the tendency of the solvent phase to interact with π- and n-electron pairs;

It follows that each of these set of system coefficients will be unique for a defined system; for example, a MCF_1+Soluble Oil system will have a set of system coefficients

different from a MCF_1+Synthetic Fluid system. The choice of MCF membrane is critical, as the resulting system should have system coefficients in **Equation 5** that are as diverse as possible in order to ***capture as many physicochemical attributes of skin*** (which cannot be reflected in a single MCF fiber) and by extension, interactions between solute and membrane. Finally, it is these system coefficients that will change in value by simply changing the "system"; i.e., changing the MCF or MWF will provide **quantitative** information on the magnitude of the formulation effect on chemical or biocide partitioning or permeability.

(iii) Formulation Effects

The following table is an example of possible additives often used in formulating a MWF formulation into one of three classes of MWF; namely, soluble oil, semi-synthetic, and synthetic MWF. There is a fourth class, straight oils, which do not require biocides additives and therefore will not be investigated in this proposal.

Soluble Oil (SO)#	*Semi-synthetic (SS)*	*Synthetic MWF (SYN)*
Naphthenic oil (70%)	***Naphthenic oil (15%)***	**pH buffer and inhibitor (5%)**
Emulsifier (17% sulfonate)	**Emulsifier (15% sulfonate)**	**EP lubricant (4%)**
Lubricant (5%)	**Emulsifier (15% alkanolamine)**	**Boundary Lubricant (5%)**
Boundary Lubricant (5%)	**Coupler (2%)**	**Boundary Lubricant (4%)**
Rust Inhibitor (3%)	**Corrosive inhibitor (6%)**	**Rust Inhibitor (10%)**
Biocide (2%)	**Biocide (2%)**	**Biocide (2%)**

Based on work performed in the pharmaceutical and cosmetics industry some or all of these additives especially the solvent base and emulsifiers/surfactants can potentially influence the dermal permeability of biocides by several physicochemical and chemico-biological interactions. Solvent effects on the epidermal membrane are believed to be associated with delipidization processes in the stratum corneum (Abrams *et al*., 1993). Not only does the presence of the solvent change toxicant disposition in skin, but also the ***percentage*** of solvent in the formulation can play a significant role in altering the partitioning and diffusion across skin. We have demonstrated this in our skin studies (Baynes and Riviere, 1998; Baynes et al., 2002b) and our more recent MCF studies as depicted by Riviere et al. (2007).

In this latter example we assessed the effects of increasing concentrations of ethanol on partitioning and diffusion of 4 solutes into the carbowax fiber. This clearly demonstrated qualitative and quantitative changes in solute disposition in the MCF. MWF formulations are often diluted and this relationship may be linear as demonstrated in our preliminary experiments and can therefore be incorporated into our interaction coefficients paradigm to predict biocide disposition in skin when the skin is simultaneously exposed to a MWF formulation of varying strengths.

Surfactants are often formulated with agrochemical and other industrial applications, and consequently serve as an additional formulation component that can alter toxicant diffusion across skin. Anionic surfactants such as sodium lauryl sulfate (SLS) can

cause swelling and disrupting of the stratum corneum and extension of the α-keratin structure resulting in spatial expansion and altered diffusion pathways for chemicals (Rhein *et al*., 1986, Scheuplein and Ross, 1970). Surfactants can also enhance solute penetration by causing fluidization of intercellular lipids in the stratum corneum (Ribaud *et al*., 1994). The dermal irritation, penetration, and enhancer effect of SLS on drug absorption has been well documented (Wilhelm *et al*., 1991) and also previously demonstrated in our earlier mixture studies in skin (Baynes *et al*., 1996, Qiao *et al*., 1996).

Researchers however often overlook the fact that *surfactants can generate micelles that retain solutes on the skin surface and effectively modulate solute penetration into skin*. Several of our recent studies strongly suggest that surfactant concentrations above or below the critical micelle concentration (CMC) can influence solute partitioning and diffusion into the MCF for four solutes interacting with the carbowax fiber in varying surfactant concentrations (Riviere et al., 2007). Note that the K_{wax} values were not significantly influenced by SLS at concentrations below the CMC, but decreased linearly beyond the CMC. These observations were part of the stimulus for this grant application that is focused on using a solvatochromatic approach (i.e., a 5-component LSER) to understanding micelle-solute interactions and how solvents modulate these interactions.

In summary, identifying and quantifying inter- molecular forces (i. e, system coefficients) influencing solute distribution behavior between different phases in the proposed chromatographic systems is another approach to assessing dermal absorption and represents a ***paradigm shift*** within which toxicologists can begin to accurately model toxicant disposition in skin following dermal exposure to complex chemical mixtures. This physicochemical approach should ***advance the science of human health risk assessment*** that currently relies on incomplete data sets and limited quantitative and mechanistic models with which to accurately predict single chemical absorption, and needless to say chemical mixtures. Our approach allows **experimental** assessment of mixture interactions in a LSER framework that previously has only been used as a mathematical tool to predict single chemical absorption.

In that same vane, we recently demonstrated (Vijay et al, 2007) that we can accurately predict ($R^2 = 0.94$) the dermal permeability of MWF biocides using an LSER approach. *In vitro* porcine skin diffusion experiments were used to first calibrate the porcine diffusion system to obtain base line system coefficients and then in separate experiments, the skin sections were exposed to 4 phenolic biocides in either a soluble oil or synthetic MWF. The figure below demonstrated significant formulations differences which are predictable within a LSER.

(iv) Selection of representative chemicals in multivariate studies.

In essence, this proposal is attempting to demonstrate quantitatively the effects of various metal-working fluid (MWF) formulations on the dermal permeability and absorption of biocides that are of occupational concern if there is dermal exposure to workers in the metal-working industry. Our approach is not to evaluate all MWF biocides but to evaluate representatives from 5 classes of frequently used biocides in the metal-working industry. Our approach initially requires calibration of the membrane-coated fiber (MCF) array of 5 diverse fibers and this calibration process requires exposing the MCF

array to a diverse series of solutes selected from a large data set (>3000 solutes) complete with the 5 molecular descriptors.

The selected biocides, training solute set, and validation solute set, with their known molecular descriptors ($\mathbf{R_2}$, π_2^H , $\Sigma\alpha_2^H$, $\Sigma\beta_2^H$, and $\mathbf{V_x}$) must have properties that are sufficiently varied to define interactions in the LSER. The objective here is to generate a large but manageable series of compounds that are orthogonal with respect to all 5 molecular descriptors; that is, there is distinct diversity in molecular descriptors. Ultimately there should be an absence of significant cross-correlation among descriptors and clustering of individual descriptor values in order to avoid generating multivariate equations that are not sensitive to changes in the chemical space. It should be recognized that with 5 molecular descriptors for over 4500 available chemicals from ADME Boxes Database (Pharma Algorithms) with known molecular descriptors, we will be sampling from a multidimensional space. Principal component analysis (PCA) is a useful chemometric technique to characterize similarities and differences between individual solutes from a large data set as proposed in this application. The primary objective is to arrive at **principle components which are essentially a** set of variables that define a projection that captures the maximum amount of variation in a data set and is thus orthogonal (i. e., uncorrelated) to the previous principle component of the same data set. PCA lends itself to identifying patterns in data to highlight similarities and differences especially in such a high dimensional data sets proposed for this project. Data sets that will receive this treatment include those solutes used to calibrate the array of fibers (also called the training or calibrating set of solutes). PCA will also be used to generate chemical clusters that will be used to validate/test the MCF array calibrated systems by evaluating goodness of fit.

The literature describes numerous techniques and algorithms to analyze or cluster high-dimensional data sets to generate a sample data set with significant molecular diversity (Yeung and Ruzzo, 2001; DeSmet et al., 2002; Fuguet et al., 2002; Gutierrez-De-Teran et al., 2002; van der Merwe and Riviere, 2006; Xu and Redman-Furey, 2007). In addition to PCA as described above, there are other techniques such as hierarchical clustering, self-organizing tree algorithm, K-means, and heuristic two-step approaches that have been successfully used to analyze gene expression profiles; chemical property profiles, and other related multidimensional data sets. However, it should be noted that in its attempt to reduce data dimensionality, this normalization technique with PCA can sometimes remove "data noise" and makes it less variable thus reducing the ability of PCA to capture data structure.

Specific Aims

The following 3 Specific Aims of the Project were

Specific Aim 1. To quantify mixture interactions influencing transport of MWF biocides between MWF formulations and the membrane coated fibers (MCF). Our main objective here is to utilize the MCF technology with LSERs to determine changes in the system coefficients (**Δ values**). These changes in system coefficients will be indicative of formulation-induced effect. This partitioning and diffusion behavior is correlated to solvatochromatic parameters and this approach will provide more qualitative and quantitative information about how MWF formulations influence biocide partitioning in

the MCF array, ultimately leading to MWF formulations that minimize biocide dermal penetration.

Specific Aim 2. ***To quantify chemical-biological interactions in a biological membrane system following exposure to MWF formulations in porcine skin flow-through diffusion cell.*** This *in vitro* biological system will be calibrated to determine system coefficients based on empirically derived dermal permeability data and molecular descriptors of the 100 selected calibration solutes. MWF formulation effects in the skin will be determined, and **changes in the system coefficients (Δ values**) in skin will be compared with those **Δ values** derived from the MCF system. The primary objective here is to evaluate the robustness of our calibrated MCF system to predict **Δ values** when skin is exposed to MWF formulations mixtures. Preliminary studies with simple solvent and surfactant mixtures suggest that this is a sound approach.

Specific Aim 3. ***To quantify the effect of MWF formulations on the in vivo dermal absorption of MWF biocides.*** The preliminary studies would have identified those MWF formulations that significantly influenced the partitioning and permeability of MWF additives based on **changes in the system coefficients (Δ values**). The primary objective here in these ***in vivo*** studies is to determine whether the significant changes in partitioning and permeability observed in the preliminary studies translate into significant changes in *in vivo permeability and ultimately*, bioavailability and maximum plasma concentrations to warrant concerns abut adverse health effects from exposure to MWF additives. This is a crucial step to validate the *in vitro* approaches.

PROCEDURES & METHODOLOGY

Specific Aim One. ***Specific Aim 1. To quantify mixture interactions influencing transport of MWF biocides between MWF formulations and the membrane coated fibers (MCF).***

This specific aim was ***modified to focus on (a) MCF Approach using PDMS as this MCF fiber has less inter-and intra-lot variability and then to focus on (b) screening of solutes for the skin absorption*** experiments in phase 2 and 3 of this project as originally proposed. We deemed this as very important to better define the chemical space within the context of chemicals that workers will be exposed to during topical exposure to MWFs.

(a) MCF Approach using PDMS fiber.

Our experiments are based on the MCF approach proposed in Xia et al. (2003). In this study, solutes were solved into a particular MWF (Metal Working Fluid) mixture. Then a membrane-coated fiber (MCF) is paced in the vial to allow the solute to partition from the solution into the MCF over a period of one to four hours. Gas chromatography and mass spectrometry are then used to extract or desorb the solute from the MCF, and the amount extracted is recorded. Differential ability of the solute to dissolve in the MCF or the MWF mixture is measured using a partition ratio (coefficient) $K_{MCF/mix}$ between the equilibrium concentration of solute in the MCF and the equilibrium concentration of solute in the working solution, our response variable is the logarithm of the partition coefficient $K_{MCF/mix}$.

According to Xia et al. 2003, the partition coefficient $\mathrm{K_{MCF/mix}}$ is calculated in the following way: consider a particular solute and a particular MWF mixture. These are combined to produce $\mathrm{V_d}$ in ml of solution at a known solute concentration of $\mathrm{C_0}$ in $\mu g/ml$ placed in a vial. The MCF (of volume $\mathrm{V_m}$ in ml) is then placed in the vial to allow the solute to partition from the solution into the MCF over a period of one to four hours. The amount of solute extracted is recorded as $\mathrm{n^0}$ in μg. $\mathrm{K_{MCF/mix}}$ is defined as

$$\mathrm{K_{MCF/mix}} = \frac{\mathrm{C_{pe}}}{\mathrm{C_{me}}} = \frac{\mathrm{n^0/V_m}}{\mathrm{C_0 - n^0/V_d}} = \frac{\mathrm{n^0 V_d}}{\mathrm{V_m(C_0 V_d - n^0)}} \quad (1)$$

Where $\mathrm{C_{pe}}$ is the equilibrium concentration of solute in the MCF and $\mathrm{C_{me}}$ is the equilibrium concentration of solute in the working solution.

Experimental conditions are defined by three factors: the solvent used to create the MWF mixture (we refer to it **as MWF formulation**); the solvent concentration used to create the MWF mixture (we refer to it as **MWF concentration**); and the **solute concentration** (we refer to it as analyte concentration). Five MWF formulations were considered: mineral oil (MO), polyethylene glycol-200(PEG-200), soluble oil (SO), synthetic oil (SYN), and semi-synthetic oil (SSYN). MWF concentrations were set at three levels: 0.05%, 0.5%, and 5%. Six analyte concentrations were considered: 0.01, 0.05, 0.1, 0.5, 1, and 5 ppm. Only a single MCF was used, namely PDMS (polydimethylsiloxane). Hence, there are 5 x 3 x 6 = 90 experimental conditions. It is quite evident that this would have been impossible to complete if multiple MCF fibers were used as originally proposed.

Under each experimental condition, all 37 solutes (see a complete list in Table 1) are ideally studied using three replicates. Unfortunately, due to a variety of reasons (e.g., lack of detection in gas chromatography, or records outside the calibration range), not all replicates are recordable, with some experimental conditions even ending in no replicates for a particular solute. Of the maximum possible 90x37x3=9,990 observations, we actually have 4,646 partition ratios.

Abraham's LFER model and the summary statistics of the data

Abraham and Martins (2004) proposed the general linear free-energy relationship (LFER) model to study the dermal absorption

$SP = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 A + \beta_4 B + \beta_5 V,$

where SP is the property of interest for the solutes, such as $logKp$, $logP$ etc, E, S, B,V and V are five solvatochromic descriptors (E, S, A, B, V) are most relevant to the solvation process during permeation (Abraham et al, 1999; Abraham and Martins, 2004). These descriptors represent different characteristics of compounds involved in the solvation process, specified as follow. E is the solute excess molar refraction, S is the solute dipolarity /olarizability, A is the overall hydrogen bond acidity, B is the overall hydrogen bond basicity, V is the McGowan characteristic volume. For most of the chemicals, V can be calculated directly, E can be obtained from experiment or calculated, A,B,S are experimentally derived. In this paper, logarithm of the partition coefficient, $logK_{mcf/mix}$, is the property of interest. The Abraham's LFER model is shown in Equation (2).

$$\log K_{mcf/mix} = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 A + \beta_4 B + \beta_5 V \qquad (2)$$

ADME Boxes 4.95, a commercial software from ACD/Labs (www.acdlabs.com) was used to identify the E, S, A, B, and V descriptors for all the 37 solutes used in the experiment. The solute names along with their descriptor values are shown in Table 1.

Table1. Descriptor values of the 37 solutes

SOLUTE	*SOLUTE Name*	*E*	*S*	*A*	*B*	*V*
1	Toluene	0.6	0.52	0	0.14	0.8573
2	Chloro- Benzene	0.72	0.65	0	0.07	0.8388
3	Ethylbenzene	0.61	0.51	0	0.15	0.9982
4	p-Xylene	0.91	0.52	0	0.16	0.9982
5	Bromo- Benzene	0.88	0.73	0	0.09	0.8914
6	Propyl-Benzene	0.6	0.5	0	0.15	1.1391
7	1-Chloro-4-Methyl-Benzene	0.71	0.74	0	0.05	0.9797
8	Phenol	0.81	0.89	0.6	0.3	0.7751
9	Benzonitrile	0.74	1.11	0	0.33	0.8711
10	4-Fluoro-Phenol	0.67	0.97	0.63	0.23	0.7927
11	Benzyl Alcohol	0.8	0.87	0.39	0.56	0.916
12	Iodo- Benzene	1.19	0.82	0	0.12	0.9746
13	Phenyl ester acetic acid	0.66	1.13	0	0.54	1.0726
14	2-chloro-Acetophenone	0.89	1.14	0	0.47	1.1363
15	Phenol, 4-methyl-	0.82	0.87	0.57	0.31	0.916

16	Nitro- Benzene	0.87	1.11	0	0.28	0.8906
17	Methyl Ester Benzoic acid	0.73	0.85	0	0.46	1.0726
18	1-chloro-4-methoxy-Benzene	0.84	0.86	0	0.24	1.0384
19	Phenylethyl Alcohol	0.81	0.86	0.31	0.65	1.0569
20	3-Methylbenzyl alcohol	0.82	0.9	0.39	0.59	1.0569
21	4-Ethyl-Phenol	0.8	0.9	0.55	0.36	1.0569
22	3,5-dimethyl-Phenol	0.82	0.84	0.57	0.36	1.0569
23	Ethyl Ester Benzoic acid	0.69	0.85	0	0.46	1.2135
24	2-Methyl-Methyl Ester Benzoic acid	0.77	0.87	0	0.43	1.2135
25	Naphthalene	1.34	0.92	0	0.2	1.0854
26	3-Chloro- Phenol	0.91	1.06	0.69	0.15	0.8975
27	p-Chloroaniline	1.06	1.13	0.3	0.31	0.9386
28	1-methyl-4-nitro-Benzene	0.87	1.11	0	0.28	1.0315
29	1-(4-chlorophenyl)-Ethanone	0.96	1.09	0	0.44	1.1363
30	3-Bromo-Phenol	1.06	1.13	0.7	0.16	0.9501
31	4-Chloro-3-Methyl-Phenol	0.96	0.96	0.67	0.38	1.0384
32	1-Methyl-Naphthalene	1.34	0.92	0	0.2	1.2263
33	Biphenyl	1.36	0.99	0	0.26	1.3242
34	Chloroxylenol	0.93	0.96	0.61	0.21	1.1793
35	4-(1,1-dimethylpropyl)-Phenol	0.79	0.8	0.5	0.44	1.4796
36	o-Hydroxybiphenyl	1.61	1.37	0.5	0.49	1.3829
37	Clorophene	1.53	1.42	0.67	0.47	1.6462

The summary statistics of the response variable $logK_{MCF/mix}$ and the predictors (E, S, A, B and V) are shown in Table 2.

Table 2. Summary statistics for response variable and descriptors.

Variable	*Minimum*	*Lower Quartile*	*Mean*	*Median*	*Upper Quartile*	*Maximum*	*Std Dev*	*N*
$\boldsymbol{logK_{MCF/mix}}$	-1.8196	0.8415	1.3288	1.3800	1.8788	3.1068	0.7190	4646
E	0.6000	0.7200	0.8661	0.8200	0.9100	1.6100	0.2180	4646
S	0.5000	0.8000	0.9020	0.9000	1.1100	1.4200	0.2203	4646
A	0.0000	0.0000	0.1195	0.0000	0.0000	0.7000	0.2312	4646
B	0.0500	0.1500	0.2993	0.2800	0.4400	0.6500	0.1491	4646
V	0.7751	0.9386	1.0575	1.0384	1.1363	1.6462	0.1705	4646

(b) **Screening of solutes for development of dermal QSARs**

A large candidate set of N=4,534 solutes was the original set, and each of these solutes have E, S, A, B, V descriptor values from the database supplied with ADME Boxes. In order to rule out the study-unfriendly solutes by using Baynes' rule in the screening process, the following additional predicted or experimental physicochemical properties were obtained from the literature or calculated: molecular weight (MW), number of hydrogen bond donators/acceptors, and octanol-water partition coefficient (logP) values.

The batch calculation option provided by ADME Boxes was used to obtain the above physicochemical properties. The batch calculation option accepts a text file containing records of SMILES (Simplified Molecular Input Line Entry Specification) strings. The

SMILES strings are chemical notations of solutes designed especially for computer storage and use [15]. The outputs of the batch calculation are the desired physicochemical properties. ADME Boxes can handle calculation for more than 1000 solutes at a time, which saves effort because only five batches need to be processed. The SMILES strings list can be generated either by ChemDraw ([16], ChemDraw is a GUI which accepts solute names and then translates them into SMILES strings) or JChem for Excel ([17], JChem for Excel takes the International Union of Pure and Applied Chemistry (IUPAC) named solutes as input and outputs SMILES strings in a batch mode).

With all nine descriptors (the five solvatochromic descriptors E, S, A, B, V plus the four physicochemical properties MW, number of hydrogen bond donators, number of hydrogen bond acceptors, and logP values), the screening process was implemented. Note that ADME Boxes provides two calculated logP values, logP (AB) and logP (ACD) and both values were used for the remainder of this work.

Screening Process

Experiments have shown that the permeation capability of certain solutes is related to certain physicochemical properties such as MW and logP,. Solutes with certain property values are more or less likely to have good permeation capability. Thus in practice empirical rules for solute screening are often used.

Lipinski et al. suggested a "rule of five" to provide an empirical way to assess potential solubility and intestinal permeation. If a solute violates any of the following criteria, then this solute is very likely to have poor intestinal absorption or permeation: 1) there are more than five hydrogen bond donors; 2) there are more than 10 hydrogen bond acceptors; 3) the MW is greater than 500 grams/mole; and 4) the logP is over five.

Lipinski's rule was proved to be successful in solute identification in intestinal absorption . However, in skin permeation, this rule may not apply. Magnusson et al. suggested a rather strict rule for predicting the solutes' potential capability of transdermal delivery. For example, they proposed that when the MW is greater than 213, then a solute will show poor permeation through dermal delivery.

Direct application of Magnusson's screening rule to our candidate set is far too stringent as it would eliminate all of the 4,534 solutes. The screening rule was therefore adjusted based on our experience, to consider only two factors: the MW and logP values. This was termed Baynes' rule. Solutes with MW equal to or greater than 400 or logP less than one or greater than four were ruled out. Because ADME Boxes provides two logP values, a solute was counted as a failure if either of the logP values violated the inclusion rule. Table 1 summarizes the difference among the above three screening rules.

Table 1: Comparison of the three Screening Rules

Predictors – connect with "and" for inclusion				
Inclusion Rule	**MW**	**logP**	**HB-a***	**HB-d****
1. Lipinski (intestinal)	<=500	<=5	<=10	<=5
2. Magnusson (transdermal)	<=213	<=1.2	<3	<0
3. Baynes (transdermal)	<400	1 <= logP <= 4	not used	not used

***: Hydrogen bond acceptors **: Hydrogen bond donors**

After applying Baynes' rule, the process was continued by removing solutes from certain chemical classes. The list of the chemical classes and the reasons for removal are summarized in Table 2.

Table 2: List of chemical classes and reasons for removal

Chemical Class	Reason for Removal
"acid"	These solutes are ionizable
"urea", "isone", "epam", "bartital"	These four classes of solutes are usually not available or not GC-amenable and require derivatization
"alol", "olol"	These two classes of solutes require derivatization

Selection criteria

To select a "representative" subset from a large molecular database, there are mainly three types of statistical optimal design approaches that have been reported in the literature: information-based designs, distance-based designs and cell-based designs.

In least squares estimation of the linear parameters of a model, the variance is proportional to the inverse of the information matrix. An information-based design considers a subset as good if some function of the variance is minimized or equivalently, some function of the information matrix is maximized. D-optimal designs are commonly used information-based designs that seek to maximize the determinant of the information matrix; they are widely used in the literature. However, this work was not primarily interested in merely estimating β_0 to β_5 of equation (1), but instead an equation (2) that has good predictive power. For this reason, D-optimality was not ideal for this work.

A distanced-based design checks the distance from a point x in p-dimensional Euclidean space R^p to a set A in that same p-dimensional Euclidean space, i.e., $A \subset R^p$. The distance $d(x, A)$ is defined as $d(x, A) = \min_{y \in A} \|x - y\|$, where $\|x - y\|$ is the p-dimensional distance in Euclidean space. U-optimal designs and S-optimal designs are two types of distance-based designs with different focus. According to Higgs [20], the U-optimal design focuses on the "coverage" of a subset that has maximum similarity to the candidate set. The U-optimal criterion is evaluated by the mean distance from each candidate point (including the design points) to the design as $\frac{1}{N}\sum_{x \in C} d(x, D)$, where N is the number of points in the candidate set, C is the candidate set, and D is the design set [12]. S-optimal designs emphasize the "spread" of the design points with an aim to get a subset in which the design points are maximally dissimilar to each other. Different from the U-optimal design, S-optimality only examines the design set. It focuses on a subset in which the harmonic mean distance from each design point to all the other points in the design is maximized [12]. The S-optimal criterion is $\frac{N_D}{\sum_{y \in D} 1/d(y, D-y)}$, where N_D is the number of design points. For both criteria, the

smaller the value the better the design. U-optimal and S-optimal are two popular designs and often serve as baselines for comparison with other newly introduced designs.

A third design class is called the cell-based design. The basic idea in the cell-based design is: for each of the k descriptors of the solutes, divide them into m bins to form m^k cells. The desired design will have at least one solute from each cell and thus the space is covered or filled. This conventional design has the problem of too many cells in high-dimensional space and a large portion of uncovered cells. Lam et al. suggested a Uniform Coverage Design to handle the "curse of dimensionality" with the aim to select a design that has the highest average uniform coverage design rate in all subspaces. Vijay et al. adopted this design to select n=25 solutes from 4,098. Though this design claimed faster exchange time and better coverage than a U-optimal design and an S-optimal design, the algorithm is rather complicated and is only seen in a non-commercial software package. Thus, this design method was not considered for the current study.

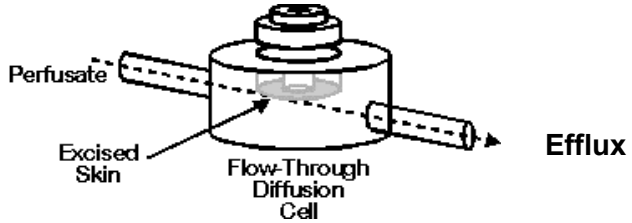
For the information-based design (D-optimal) and the distance-based designs (U-optimal and S-optimal), implementations are available from SAS 9.2 [22] with the OPTEX procedure. The U-optimal design was chosen because it adequately fit the purposes of this study—to select a set that is diverse, covers the space well, and has maximal similarity to the candidate set. In contrast, D-optimal designs seek to minimize the variance of the regression parameters and S-optimal designs seek to maximize the spread of the design points.

Specific Aim 2. To quantify chemical-biological interactions in a biological membrane system following exposure to MWF formulations in porcine skin flow-through diffusion cells..

We conducted numerous in vitro flow-through Diffusion Cell system experiments to assess formulation effects on the dermal absorption of more than 35 solutes. Below is a description of these in vitro diffusion experiment.

Flow-through diffusion studies

These *in vitro* experiments were be conducted using porcine skin sections inserted in the Bronaugh flow-through diffusion cell **as depicted below**. Our flow-through diffusion cell system uses flow-through diffusion cell blocks as described by Bronaugh and Stewart (1985)**.**



An illustration of an *in vitro* flow-through diffusion cell. These diffusion cells are placed in a 14-cell heated block and maintained at 37°C.

Skin from the abdomen of female weanling Yorkshire pigs was carefully excised and dermatomed to a thickness of 500 μm using a Padgett dermatome. Circular skin sections were placed epidermal side up into each Teflon flow-through diffusion cell to

provide a dosing area of 0.64 cm². The dermal side in each cell was bathed with receptor fluid (Krebs-Ringer bicarbonate buffer containing albumin and glucose with pH=7.4) at a set flow rate (4ml/hr) by a multichannel peristaltic cassette pump. The receptor fluid mimics *in vivo* plasma and facilitates absorption of lipid soluble solutes. Perfusate was collected at 0.25 hr intervals for the first 2-hr and 1-hr intervals thereafter for 8 hrours and every 4 hours until 24 hours. Chemicals were assayed in perfusate over time using our developed micro-fiber separation technique (Xia and Leidy, 2001; Mohammed *et al*., 2003). In brief, the MCF was immersed in micro-filtered perfusate samples, and then directly injected into the injection port of the GC-MS for analysis.

Data processing and statistical analysis.

At least 6 replicates per mixture or treatment group were conducted for all flow-through diffusion cell studies. All real-time data was handled by our custom copyrighted computer database to facilitate data analysis. Solute p*ermeability* (cm/hr) of in this diffusion cell system was determined from the following equation:

Permeability (cm/hr) = Flux ($\mu g/cm^2/hr$) / Dose ($\mu g/cm^3$)

where steady state flux of the solute was determined from the steady state slope derived from a plot of cumulative solute flux vs time.

The focus of this project is formulations research and the experimental design was aimed at deriving interactions coefficients and statistically determine the effects of different MWF formulations on the system coefficients. Multi-level interactions among these chemical components in the mixtures could potentially occur and be identified statistically. General Linear Model procedure with LSD multi-comparisons test with significance level at α = 0.05 was conducted to statistically determined differences across formulations, concentrations, and the 12 biocides in these analyses. All analyses were carried out using SAS software (SAS Institute Inc., Cary, NC). Confidence intervals and *p*-values for the main and interaction effects were derived and graphical methods such as compass plots was used to display changes in mean values.

Specific Aim 3*. *To quantify the effect of MWF formulations on the in vivo dermal absorption of MWF biocides.

It would not be economically feasible to evaluate the *in vivo* absorption of all test (validation) solutes including the 12 biocides in all of the MWF formulations and concentrations tested in specific aims #1 and #2. However, data from these pervious experiments allowed us to target a biocide used in MWF formulations that resulted in synergistic or antagonistic changes in biocide permeability and validate these permeability changes *in vivo*.

We therefore focused on the phenolic biocide, ortho-phenylphenol OPP), which is used in MWFs as a biocide. Control pigs will be exposed to the biocide alone to better assess changes in the biocide pharmacokinetic parameters primarily due to the MWF formulation. Recall that the MCF and *in vitro* skin experiments required exposure to solutes and biocides in water only. Intravenous studies were also required for two primary reasons; namely, to more accurately assess the elimination half-life and thus better optimize the design of the dermal exposure studies and to obtain baseline AUC values that can be used with the dermal AUC values to calculate the absolute bioavailability of each of the biocides.

These studies were randomized cross-over design with 4 replicates in each treatment group. Based on above stated limitation, we assessed dermal absorption in the following 4 MWF formulations: water only; soluble oil; semi-synthetic fluid, and synthetic fluid. Animals had jugular catheters placed to facilitate blood draws and be acclimatized for this procedure. Jugular catheters were placed using the non-surgical technique. During the protocol, animals were housed separately in individual farrowing crates to prevent catheter displacement and destruction. Once the catheter was placed, the dorsum of the pig will be clipped prior to application of the MWF formulation to the surface of the skin in customized Hill-Top chambers. This is not analogous to patch testing small areas for skin testing, but with rather larger surface areas (e.g., 5 cm x 20 cm) along the dorsum of the pig with modified Hill-Top chambers that will result in measurable solute concentrations in the blood samples**.** These were 5-day exposures represent worse case exposure scenarios and the skin surface was swabbed with a soapy solution at 5 days to terminate surface exposure. Blood samples were collected at frequent intervals (0.25, 0.5, 1, 2, 4, 8, 12, 18 hr) during the first 24 hours after topical exposure and every 12 hours thereafter. Because of concerns about "flip-flop" kinetics for several of the MWF formulations, these studies were conducted for at least 10-12 half-lives to ensure that sufficient data is captured during the absorption and depletion phase in the animal. Animals were euthanized at the completion of the studies and all tissues were harvested for sample analyses.

Calculations and Pharmacokinetic Analyses:

Biocide plasma concentration – time graphs were plotted on a logarithmic y-axis to ascertain the relationship (linear vs. nonlinear) between concentration and time. Pharmacokinetic (PK) analysis were performed with WinNonLin (Pharsight, Mountain View CA) using compartmental and non-compartmental modeling approaches. PK parameters wiere determined based on an individual pig basis. Parameters that were calculated using standard equations include absorption rate constant (Ka), area under the curve (AUC), absolute dermal bioavailability (F); maximum plasma concentration (C_{max}), steady state volume of distribution (Vss), absorption and elimination half-life ($t_{1/2}$), mean residence time (MRT), and clearance (Cl). All values were then averaged across pigs and their standard errors calculated**.** Residuals from the predicted pooled model were calculated and plotted to determine whether there is bias and if the variance is relatively constant. Model predictions versus observed plasma concentrations were graphed to exhibit model goodness of fit

Statistical analyses were focused on discerning formulation effects attributed to each of the 3 classes of MWF formulations and within the context of the randomized cross-over experimental design. General Linear Model procedure with LSD multi-comparisons test with a significance level of $\alpha = 0.05$ was conducted to statistically determined differences across formulations. All analyses were carried out using SAS software (SAS Institute Inc., Cary, NC).

RESULTS & DISCUSSION

<u>Specific Aim 1.</u> To quantify mixture interactions influencing transport of MWF biocides between MWF formulations and the membrane coated fibers (MCF).

In this specific aim we used several approaches to assess mixtures interaction: (a) determine formulation effects using MCFs and (b) determine a method to screen a large solute database that will be suitable for development of more robust QSARs relevant to MWF formulations.

(a) MCF Studies

The study of dermal permeation, membrane-coated fiber (MCF) array (Xia et al. 2003) was our originally proposed approach in which the molecular interactions are simulated by multiple MCFs. This was the basis for the proposed project. The partition coefficient of a solute among the MCF and the solution was determined and used as a measurement indicating the permeation capability of that solute. Abraham LSER model is one of the most popular quantitative structure-activity relationship (QSAR) models that been used in the quantitative analysis of skin permeation data. However, when the experimental conditions get complicated, it's not always appropriate to use Abraham's model with a single set of regression coefficients. In this project, we decided to use a single MCF, the PDMS fiber that most represents skin, and extended the one-factor expanded Abraham model proposed in Xu et al. 2013 to three factors, that not only accounts for complex experimental conditions but also improve the ability to conduct the rigorous hypothesis testing. In summary, the analysis results demonstrate that the expanded Abraham model not only has a better model fit and predictive power, but also the power of testing various scientific hypothesis. This project also studied the partition theory (Xia et al. 2003), the analysis reveals that the partition theory is valid only under certain assumptions. In essence, this study suggests that increasing the MWF tank-side concentration decreases the absorption of highly lipophilic compounds and the more concentrated the MWF the more lipophilic the environment and the more likely for those chemical solutes with high Ko/w to remain in the MWF and not absorb into the fiber.

Insufficiency of Abraham's LFER model and Expanded model

While Abraham's model in Equation (2) is simple and easy for interpretation, it not always sufficient, especially for large datasets under complicated experimental conditions.

Xu et.al 2013 has shown the insufficiency of Abraham's LSER model, such as the significantly different group means, different regression coefficients under various experimental condition, poor fit statistics and predictive power etc. With three factors (MWF formulation, five levels; MWF concentration, three levels; analyte/solute concentration, six levels) and a total of 90 different experimental conditions, our experiment setting is much complicated than the one in Xu et. al 2013, which only consists of a single factor (MWF formulation, four levels) and four experimental conditions, thus we would expect a single LSER model will be even difficult to meet the complication. For example, the insufficiency of one single LSER model can be demonstrated by the following boxplot of $logK_{MCF/mix}$ grouped by analyte concentration. As the analyte concentration increases, the logarithm of partition coefficient decreases, which indicates that when the analyte concentration increases, the analyte is more likely to stay in the MWF mixture rather than penetrate into the MCF fiber.

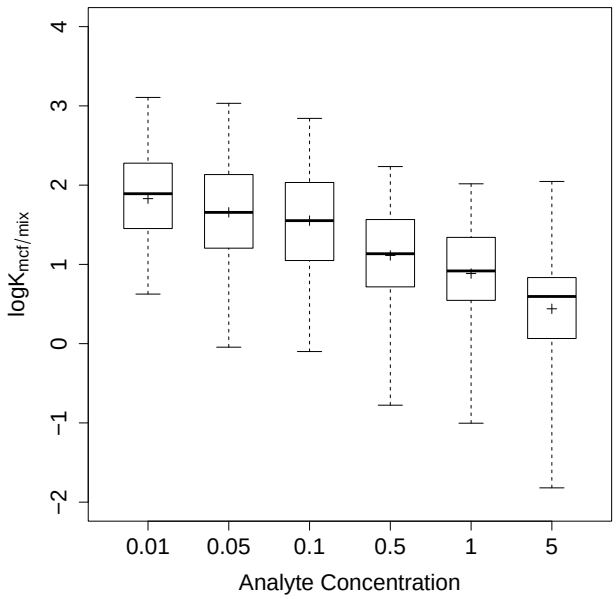


Figure 1 Boxplot of $logK_{MCF/mix}$ across different analyte concentrations

However, the above Figure 1 is the overall effect of analyte concentration, not accounting for the effect of MWF formulation and MWF concentration. Thus a more detailed visualization was desired. The Figure 2 below depicts the trend of logKmcf/mix over analyte concentration in all 15 MWF formulations and MWF concentration combinations. It shows a similar trend as in Figure 1. Figure 2 suggests that instead of viewing analyte concentration as a third factor across MWF formulation and MWF concentration, we can take it as a (numerically) nested factor within each of the MWF formulation and MWF concentration combination. By doing this, we place a structure within each MWF formulation x MWF concentration condition, and may be able to see how logKmcf/mix change over analyte concentration numerically.

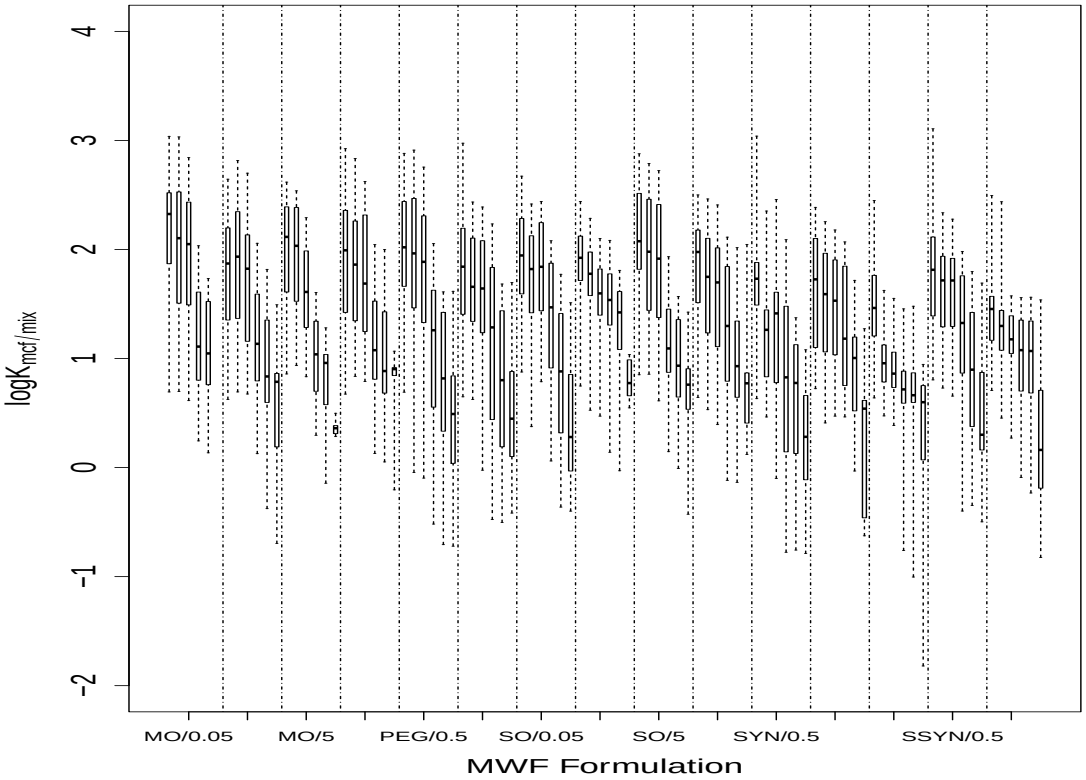


Figure 2. Boxplot of logKmcf/mix over analyte concentration in each MWF formulation and MWF concentration combination. The 15 experimental conditions (MWF Formulation/MWF concentration) from left to right are: MO/0.05, MO/0.5, MO/5, PEG/0.05, PEG/0.5, PEG/5, SO/0.05, SO/0.5, SO/5, SYN/0.05, SYN/0.5, SYN/5,SSYN/0.05, SSYN/0.5, SSYN/5.

We proposed a new nested model as in Equation (1).

$$logK_{mcf/mix,ijl} = F_{1jl}C_{i1l}(\beta_{011} + \beta_{111}E_l + \beta_{211}S_l + \beta_{311}A_l + \beta_{411}B_l + \beta_{511}V_l + \beta_{611}t_l) + F_{1jl}C_{i2l}(\beta_{012} + \beta_{112}E_l + \beta_{212}S_l + \beta_{312}A_l + \beta_{412}B_l + \beta_{512}V_l + \beta_{612}t_l) + \ldots F_{5jl}C_{i3l}(\beta_{053} + \beta_{153}E_l + \beta_{253}S_l + \beta_{353}A_l + \beta_{453}B_l + \beta_{553}V_l + \beta_{653}t_l) \quad (1)$$

Where $logK_{mcf/mix,ijl}$ is the l^{th} observation from MWF formulation i, MWF concentration j, t_l is the logarithm (base 10) of analyte concentration of the l^{th} observation, β_{dij} is the regression coefficient of "descriptor" d (d=0 for intercept, d=1 for E, d=2 for S, d=3 for A, d=4 for B, d=5 for V, d=6 for analyte concentration effects correspondingly) for MWF formulation i and concentration j. We took the logarithm of analyte concentration as it is a common way in practice.

This analyses suggest that the nested model of Equation (1) is a good approximation to the full cross-factor model. But the nested model has both less number of regression coefficients and higher leave-one-solute-out Q^2, which indicates when applying to new

dataset, the nested model is more predictive.

Table 1. Fit statistics of simple model, expanded cross-factor model and nested model.

Statistics	*Simple Model*	*Expanded cross-factor Model*	*Nested Model (1)*
N	4646	4646	4646
r^2	0.59	0.89	0.86
Adj-r^2	0.59	0.87	0.86
Q^2_{LOO}	0.59	0.86	0.86
Adj-Q^2_{LOO}	0.56	0.55	0.80

We can intepret the condensed model of Equation (1) in more details as follows with the 15 rows in Equation (1), each representing the regression function for one MWF formulation and MWF concentration combination. Each row has a set of partial slopes and each varies among the different MWF formulation and MWF concentration combinations. For example, the partial slope of E decreases as the MWF concentration increases within each MWF formulation. In minearl oil, the effect (sign of beta1) of E (solute excess molar refraction) even changes as the MWF concentration increases. To be specific, using MWF formulation of mineral oil at concentration 0.05, if we increase a unit in solute excess molar refraction and other predictors fixed, the partition coefficient will increase (the 95% confidence interval lays above the reference line). We also demonstrated that increased levels of hydrogen bond acidity are associated with decreased partition coefficients. However, the pattern of decrease changes according to the concentration of MWF. For example, in both MO and SO, higher MWF concentrations result in smaller decrease in partition coefficients. Another analysis indicates that increased level of hydrogen bond basicity generally leads to decreased partition coefficients, except for soluble oil at concentration 5, where hydrogen bond basicity has no significant impact on the partition coefficient. Finally, larger molecular or molar volume tends to have larger partition coefficient. In SO, SYN and SSYN, when MWF concentration increases, the change in partition coefficients decreases.

Validation of Partition Theory

According to Xia et.al. 2003, it is assumed that the amount of solute extracted n^0 is proportional to the analyte concentration C_0, which indicates that as the analyte concentration increases, there will be more (in proportion) analyte partitioned into the MCF fiber. Based on this assumption, suppose the analyte partitioned into the MCF fiber is $n^0 = pC_0$, where p is a constant, 0<=p<=1, by Equation below:

$$K_{MCF/mix} = \frac{n^0 V_d}{V_m(C_0 V_d - n^0)} = \frac{pC_0 V_d}{V_m(C_0 V_d - pC_0)} = \frac{pV_d}{V_m(V_d - p)}$$

This Equation suggests $K_{MCF/mix}$ is independent of C_0, which means no matter what the solute (analyte) concentration is, the partition coefficient remains the same. This is called "partition theory". If this partition theory holds true, it has practical meaning in

metalworking industry. It was interesting to determine whether this partition theory is supported by our data.

With the nested model in Equation (1), to test whether partition theory holds, it is equivalent to test whether the effect of analyte concentration is insignificant wtih the hypothesis:

$$H_0: \beta_{6ij} = 0 \text{ for all i=1,2,3,4,5 and j=1,2,3}$$

With the full data set, the p-value for H_0 is less than 0.001. This infers that in at least one MWF formulation and MWF concentration combination, the analyte concentration effect is signficiant. In fact, the indivudal P-values for testing each $\beta_{6ij} = 0$ show that, ***only in MO/0.05, PEG/5 and SYN/0.05, the analyte concentration effect is insiginficant.*** For other conditions, the analyte concentration effect is signficant and thus partion theory is violated. For example, in mineral oil at concentration 0.5, with one unit increase in log analyte concentration and other predictors fixed, the log partition coefficient will decrease one unit. Both MO and SO show decreasing trend as the MWF concentration increases. But only MO at concentration 0.05 has a positive partial slope for analyte concentration.

(b) Solute Screening studies

Initially we were able to use a statistical approach known as U-optimzation to select 32 training solutes and 32 validation solute sets for these QSARs. We also added at least 11 amines and phenolic biocides that are relevant to current additives use in the MWFs. We also completed MCF exposure experiments with the phenolic biocides and we were able to demonstrate that there were discernable interactions when the biocides were applied together.

The goal of any design-of-experiments activity is to identify a training set that is "better than" other training sets that were either arbitrarily created or created using alternative methods. A practical and transparent procedure was applied to select diverse and representative training and validation sets as summarized in the work flow (Figure 1 below). Two steps were carried out based on the original large candidate set. The first step was the screening process, which removed solutes with possible poor permeability by "Baynes' rule" and unavailable or extremely toxic solutes by identification of their chemical suffix. The second step was to select training and validation sets using the U-optimal criterion. Principal component analysis showed that the proposed procedure selected solutes representative of the solute space. Another merit of the procedure was the ease of use of the standard U-optimal design using SAS software. Though the fit statistics R^2, internal and external predictive power measurements Q^2_{LOO} , Q^2_{EXT} of the LFER model built using the U-optimal training set (as shown in Table 8) are not impressively high, which means more predictors may be needed in the LFER model, the other aim here was to stay true to the Abraham LFER model as much as possible due to its popularity in modeling dermal permeability. There are many other LFER models available and many of them have different descriptors than the Abraham LFER model. A model that better fits the could have been developed,a but this was not the purpose of the paper. Moreover, the statistics in Table 8 are better than those of the LFER model built upon the non U-optimal Vijay's set [1] in Table 9.

This study suggests several avenues of possible future improvement. The screening process is critical. Ideally, screening should occur once with the subsequently identified training and validation sets being those solutes used for experimentation. In this study, it was not possible to anticipate the many practical limitations that resulted in redefining the screened set and ultimately using a training set of only 21 solutes when the original intent was to use 32 solutes. Despite these difficulties, the steps proposed in this phase of the project are repeatable and effective for principled identification of training and validation sets starting from large candidate sets.

This work was the basis for further experiments using in vitro porcine skin with the augmented validation set. As expected, the resulting permeation coefficients (logKp) added to the robustness of the LFER models as described in specific aim 2 described below.

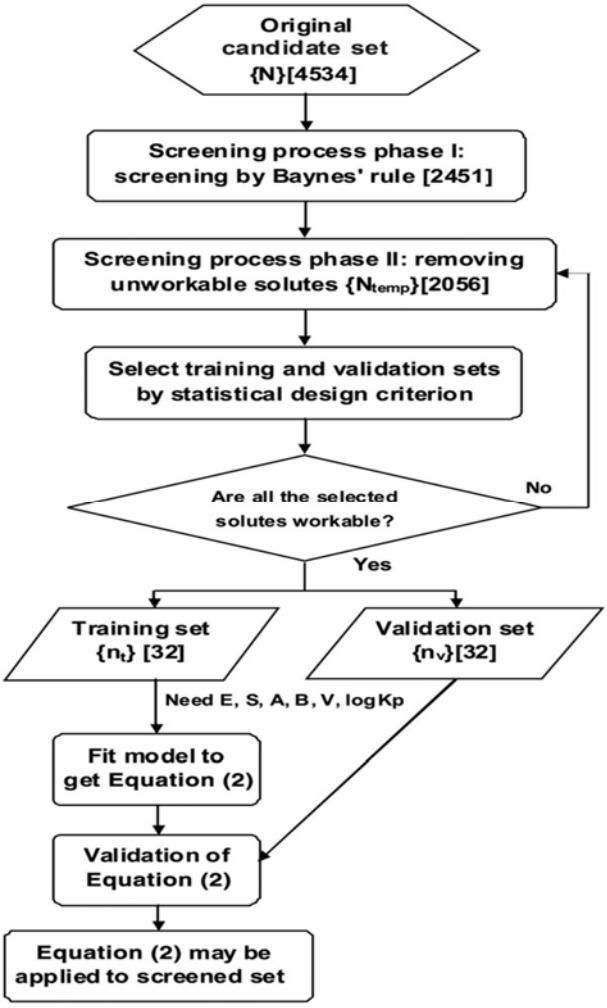


Figure 1. Work Flow. The character in {} is general notation for the size of the resulting dataset. The character in [] represents size of the resulting dataset in the application discussed in this work. Equations 1 and 2 are provided below.

$$\log Kp = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 A + \beta_4 B + \beta_5 V \quad (1)$$

$$\log Kp = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 A + \beta_4 B + \beta_5 V \quad (2)$$

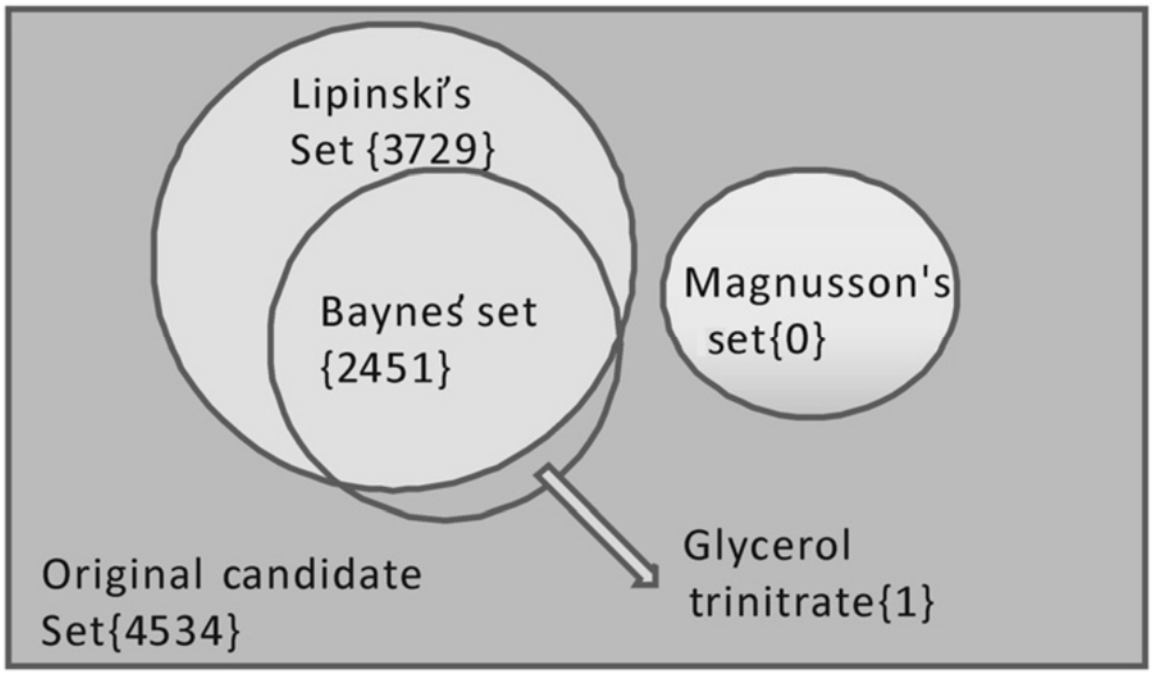


Figure 2. Screened results of the three inclusion rules. The character in {} represents the size of the resulting set.

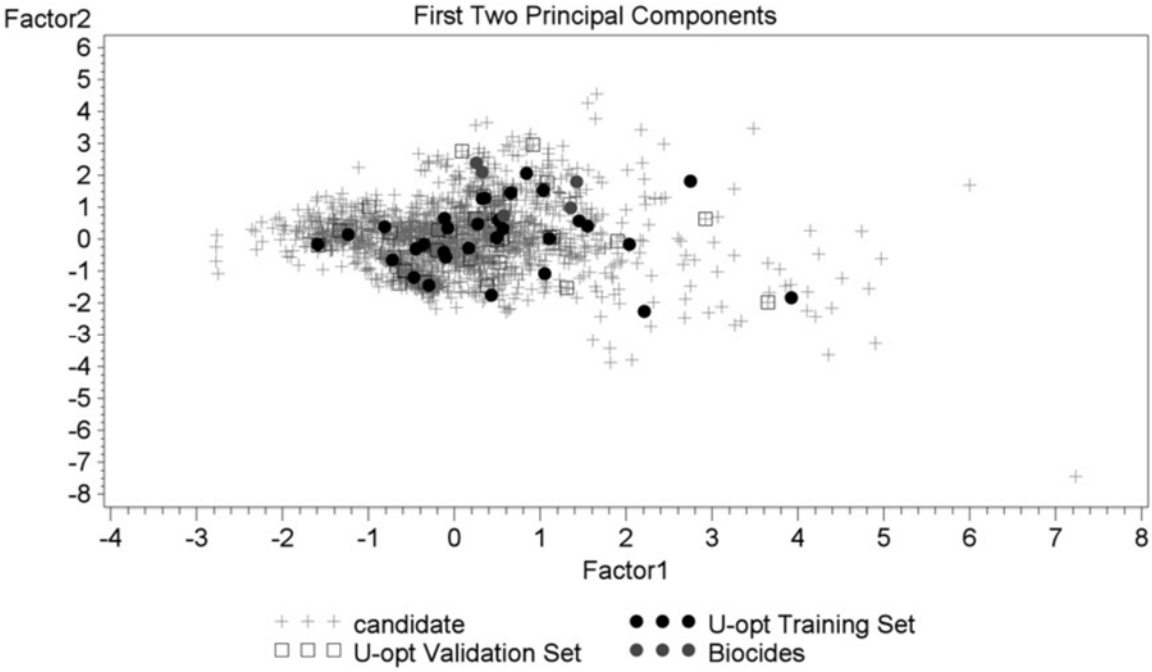


Figure 3. Scatterplot of first principal component (x axis) vs. second principal component (y axis) of U-optimal, S-optimal and D-optimal training sets each of size 32, relative to the screened set of size 2056. The black dots are U-optimal training set solutes, the open squares are S-optimal training set solutes and the grey dots are D-optimal training set solutes. Note: The D-optimal set only has 10 unique solutes, of which six overlapped with the S-optimal set.

Specific Aim 2. To quantify chemical-biological interactions in a biological membrane system following exposure to MWF formulations in porcine skin flow-through diffusion cells..

The first objective of this specific aim was to (a) assess skin diffusion of a diverse series of solutes based on solute selection work conducted in specific aim 1 as proposed. This generated robust QSARs that have direct application to protecting workers' health. The second objective was to (b) focus on a series of amines with different chemistries and are used as biocides and also serve as performance additives during the metal machining process. The use of these amines are a growing concern in the metalworking industry.

(a) Development of Dermal QSAR models for MWF formulations

Our results suggest that when the experimental conditions are complex, for example when there is more than one type of MWF formulation, an expanded model that accounts for the heterogeneity across the MWF formulations will greatly improve model fitting and predictive power. Skin permeability was shown to have the rank order: water > synthetic > semi-synthetic > soluble oil. Please see figure below. Additionally, fitted relationships between permeability and solute characteristics differ according to solvents. We demonstrated that the expanded model (r^2 = 0.70) improved both the model fit and the predictive power when compared with the simple model (r^2 = 0.21).

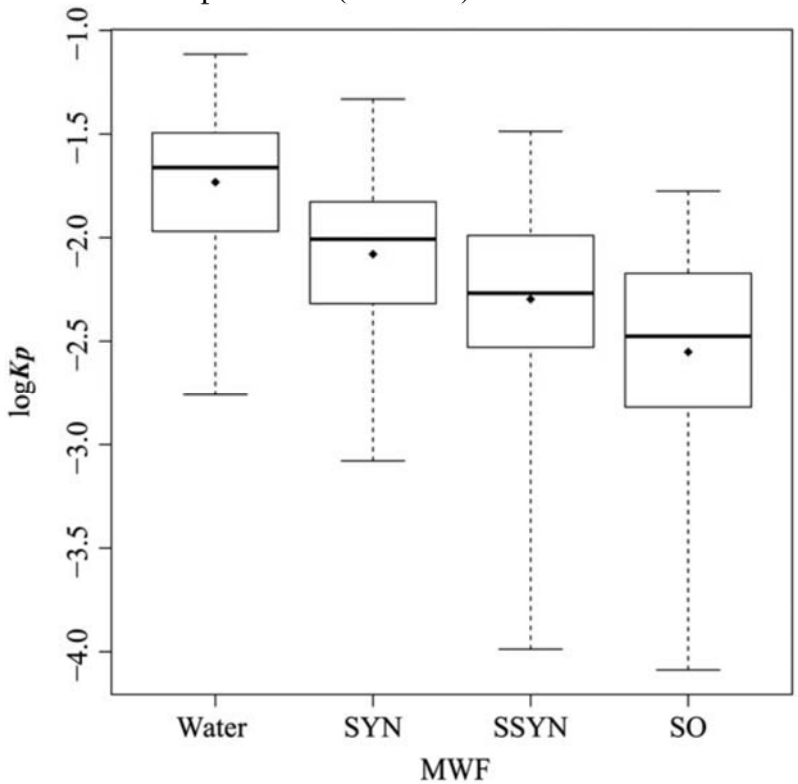


Figure. 1 Boxplot of log Kp across MWF formulations of the validation set. Numbers of observations in each MWF are: 140 (water), 105 (SYN), 138 (SSYN) and 103 (SO). The mean permeability follows the order: water > SYN > SSYN > SO. The five lines from top to bottom of each boxplot represent: maximum, 3rd quartile, median, 1st quartile and minimum. The diamond represents the sample mean.

Fitted simple model and expanded model

Instead of fitting one simple model for each experimental condition, we developed an expanded version of Abraham's LSER model to adjust for the heterogeneity introduced by various MWF formulations. Abraham's LFER model is shown in Equation (1).

$$\log Kp = \beta_0 + \beta_1 E + \beta_2 S + \beta_3 A + \beta_4 B + \beta_5 V \quad (1)$$

As is clear in Equation (1), this model makes no allowances for differences due to experimental conditions; predictions of log Kp remain the same, irrespective of experimental conditions. This insufficiency was addressed in this project.

In the expanded model of Equation (2), the partial slopes of E, S, A, B, and V are allowed to differ according to MWF formulations:

$$\begin{aligned}\log Kp_{i,j} \;=\; & F_{1j}\left(\beta_{01} + \beta_{11}E_j + \beta_{21}S_j + \beta_{31}A_j + \beta_{41}B_j + \beta_{51}V_j\right) \\ & + F_{2j}\left(\beta_{02} + \beta_{12}E_j + \beta_{22}S_j + \beta_{32}A_j + \beta_{42}B_j + \beta_{52}V_j\right) \\ & + F_{3j}\left(\beta_{03} + \beta_{13}E_j + \beta_{23}S_j + \beta_{33}A_j + \beta_{43}B_j + \beta_{53}V_j\right) \\ & + F_{4j}\left(\beta_{04} + \beta_{14}E_j + \beta_{24}S_j + \beta_{34}A_j + \beta_{44}B_j + \beta_{54}V_j\right) \end{aligned} \quad (2)$$

It is possible that some parameters in Equation (2) are not significant, thus sometimes a more condensed model is attainable. To be specific, to get a more condensed model, non-significant parameters in Equation (2) were removed and a lack-of-fit F-test was performed. If the F-statistic of the lack-of-fit test is not significant, the final condensed model will be defined.

SAS 9.3 and R were used for data analysis. The simple Abraham model in Equation (1) was fit and Equation (3) was obtained, where the numbers in parentheses are standard errors,

$$\widehat{\log Kp}_j = -0.78(0.19) + 0.35(0.12)E_j - 0.35(0.11)S_j - 0.02(0.11)A_j + 0.48(0.19)B_j - 1.47(0.23)V_j \; . \quad (3)$$

The above expanded model was then fit to produce Equation (4) below:

$$\begin{aligned}\widehat{\log Kp}_{i,j} = & \\ & F_{1j}\left(-1.14(0.22) + 0.92(0.14)E_j - 0.78(0.12)S_j + 0.03(0.12)A_j - 0.77(0.22)B_j - 0.39(0.28)V_j\right) \\ & + F_{2j}\left(-0.55(0.24) + 0.71(0.16)E_j - 0.62(0.14)S_j - 0.20(0.15)A_j - 0.28(0.25)B_j - 1.28(0.31)V_j\right) \\ & + F_{3j}\left(-0.92(0.27) - 0.01(0.17)E_j - 0.24(0.15)S_j - 0.30(0.16)A_j + 1.40(0.26)B_j - 1.67(0.33)V_j\right) \\ & + F_{4j}\left(-0.84(0.26) - 0.46(0.14)E_j + 0.20(0.14)S_j + 0.10(0.13)A_j + 2.00(0.25)B_j - 2.21(0.29)V_j\right). \end{aligned} \quad (4)$$

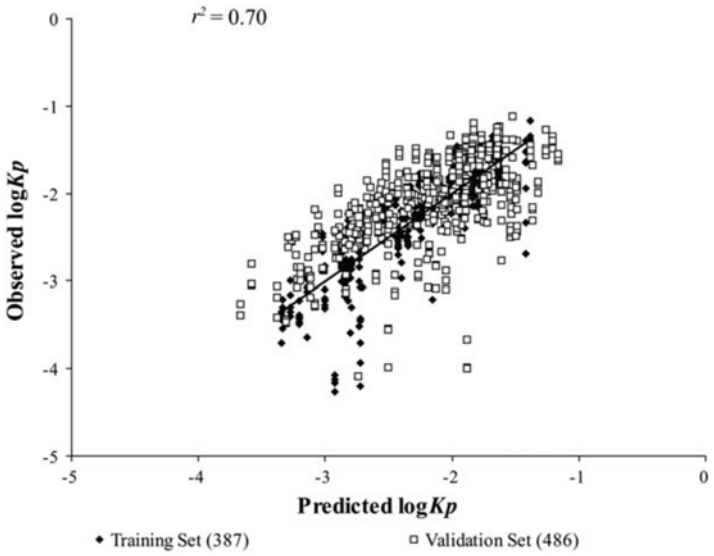


Figure 2. Observed vs. predicted log Kp of the training set and the validation set for the expanded model of Equation (4).

Each row in Equation (4) represents the fitted partial slopes for one MWF formulation.

By removing the insignificant coefficients from a simple and also expanded Abraham LSER model, we obtained the condensed model as described in the Equation below::

$$\widehat{\log Kp}_{i,j} =$$

$$F_{1j} * -0.77(0.16) + F_{2j} * -1.08(0.16) +$$

$$F_{12j} * \left(0.83(0.10)E_j - 0.71(0.09)S_j - 0.53(0.17)B_j - 0.79(0.21)V_j\right) +$$

$$F_{3j} * \left(-0.92(0.27) - 0.25(0.11)S_j - 0.30(0.16)A_j + 1.41(0.25)B_j - 1.67(0.31)V_j\right) +$$

$$F_{4j} * \left(-0.83(0.25) - 0.32(0.10)E_j + 2.07(0.25)B_j - 2.16(0.28)V_j\right)$$

where F_{12j} =1 if the jth observation is from MWF formulation 1 (water) or 2 (SYN), F_{12j}=0 otherwise.

To test the sufficiency of the condensed model in above Equation, a lack-of-fit test was performed. The SSE and degrees of freedom of the reduced model (i.e. condensed model) and the full model (i.e. expanded model in Equation 4) were assessed. The F statistic is 1.5425 with a p-value of 0.1312. Since the p-value is larger than 0.05, we regard the condensed model in above Equation as sufficient.

It is also possible to collapse the coefficients of SSYN and SO first, but the lack of fit test produced a significant p-value of 0.0187, thus we take the model in above Equation as the final condensed model

Regression statistics for the condensed model above.

Statistics	*Condensed model*
n	387
RMSE	0.32
r^2	0.69
$adj\text{-}r^2$	0.68
Q^2_{LOO}	0.67
$Q^2_{LOO\text{-}adj}$	0.41
Q^2_{EXT}	0.31

Our choice of using separate training and validation sets emanate from the fact that it is considered the standard practice in QSAR studies concerning dermal permeability (see OECD guidelines on QSAR model validation). Irrespective of whether an external validation set is used for model assessment, any internal validation steps should be conducted using a leave-one-solute-out approach, as we have presented here. According to the above Equation, the effect of the MWF formulation (i.e. solvent) is not as simple as an additive or linear effect. Given the increasing amounts of oil content of the varying formulations, from water (no oils), to SYN (no mineral oil but other oils), to SSYN (moderate mineral oil), to SO (heavy mineral oil), one may have easily predicted the observed ordering of permeability across the various MWF formulations. The model however, implies much more than this predictable ordering. As we change the MWF formulation, permeability coefficients do not change in a simple linear additive fashion. In other words, permeability coefficients for water are not simply a constant increase above permeability coefficients for soluble oil. Instead, the structure of dependence between permeability and solute characteristics changes with the changing MWF formulations, and this is reflected in different coefficients for each MWF formulation group. For example, the coefficient for B (hydrogen bonding basicity) is 2.07 in soluble oil, 1.41 in semi-synthetic oil and -0.53 in both water and synthetic oil. Recalling the interpretation of coefficients in linear regression, a coefficient of 2.07 for B in soluble oil means that a one unit increase in overall hydrogen bond basicity of a solute will increase log Kp by 2.07 units (thus increasing permeability by a factor of $10^{2.07} = 117.5$) provided all other solute descriptors are held fixed. This represents a very large impact of a solute's hydrogen bond basicity. In contrast, when either water or synthetic oil are used as the solvent, a one unit increase in overall hydrogen bond basicity will decrease log Kp by 0.53 unit (thus decreasing permeability by a factor of 10-0.53 = 0.295). Both the directional change (increase versus decrease) and the factor of change (above 100 times versus less than 1/3) are relevant for understanding the mechanisms of permeation in the various MWF formulations.

In conclusion, this study demonstrated how a QSAR model with 5% MWF information can be used to predict changes in skin permeability according to the MWF formulation. For example, a 95% lower confidence bound of 0.5225 for the difference between log Kp from water-based or synthetic oil-based MWF formulation and log Kp from soluble oil fluids suggests that workers should be more concerned about dermal absorption of formulation components in water-based and synthetic MWFs than soluble oil fluids. In fact, the lower confidence bound implies that permeability in water or synthetic oil is likely

to be greater than $10^{0.5225} = 3.33$ times the permeability in soluble oil, and this information may be highly relevant for controlling exposure and consequential health effects in workers.

(b) MWF formulation effects on skin diffusion of amines

The focus of one of our in vitro studies relates to the permeability of six amino-based additives that are often used as biocides and corrosion inhibitors in MWFs. Amines are widely used in MWFs because these molecules are able to solubilize water-insoluble materials, neutralize acids, buffer the MWF pH to a desired level (usually between 8.5 and 9.5) and prevent rust formation. The machines and metal parts are in contact with water so it is necessary to add corrosion inhibitors to protect them and neutralize the corrosive agents. Some amines can also be used as biocides to control the growth of bacteria or fungi. Therefore, the amines are a very interesting and beneficial group of chemicals that serve multiple important functions in MWFs. For this reason, our laboratory decided to focus its research on six of these amino-based corrosion inhibitors and biocides and more precisely on four ethanolamines and two cyclic amines.

To determine the absorption of six amines used as corrosion inhibitors and biocides in MWFs, porcine skin flow-through diffusion cell experiments were conducted with hydrophilic ethanolamines (mono-, di- and triethanolamine, MEA, DEA and TEA respectively) and a mixture of lipophilic amines (dibutylethanolamine, dicyclohexylamine and diphenylamine). The six amines were dosed in four vehicles (water and three generic water-based MWF formulations) and analyzed using a scintillation counter or gas chromatography/mass spectrometry. These 24 h studies showed that dermal absorption significantly ($P<0.05$) increased from water for the six amines (e.g. 1.15 ± 0.29% dose; DEA in water) compared to other formulations (e.g. 0.13 ± 0.01% dose; DEA in semisynthetic MWF) and absorption was greatest for dibutylethanolamine in all the formulations. The soluble oil formulation tended to increase the dermal absorption of the hydrophilic amines. The permeability coefficient was significantly higher ($P<0.05$) with TEA relative to the other hydrophilic amines (e.g. $4.22 \times 10^{-4} \pm 0.53 \times 10^{-4}$ cm h^{-1} [TEA in synthetic MWF] vs. $1.23 \times 10^{-4} \pm 0.10 \times 10^{-4}$ cm h^{-1} [MEA in synthetic MWF]), except for MEA in soluble oil formulation. The significant differences seen between the two groups of amines might be explained by the logarithm of the octanol–water partition coefficient (logKow or logP). Indeed, the logKow of MEA, DEA and TEA are all close to –1 (respectively –1.06, –1.08, –1.05) whereas DBEA, DCHA and DPA have logKow of 2.86, 3.69 and 2.97, respectively (logKow estimation by ACD/Percepta, ACD/Labs 2012 release, Advanced Chemistry Development, Inc.). These latter three compounds are less soluble in water and can more readily cross the skin compared to the three hydrophilic molecules that are more likely to stay in the water vehicle.

Other studies in our lab have demonstrated that the permeation is higher in synthetic, semisynthetic and finally in soluble oil type of MWFs (Vijay et al., 2009). We found this absorption pattern with the three lipophilic amines but not with the three hydrophilic ethanolamines. There was significanty great skin retention and penetration (skin + absorption) of the hydrophilic ethanolamines from the topical soluble oil formulation than from the synthetic and semisynthetic formulations; that is, for MEA, DEA and TEA: SO>SS = SYN. When an ANOVA was run on just the three Water-based MWFs with the lipophilic amines, without the water doses, the differences seen in the absorption

and permeability of the three lipophilic amines followed a converse pattern to the hydrophilic ethanolamines, but were not always significant: (DCHA: SYN>SS, SO); (DPA: SYN>SS ≥ SO); (DBEA: SS ≥ SYN ≥ SO).

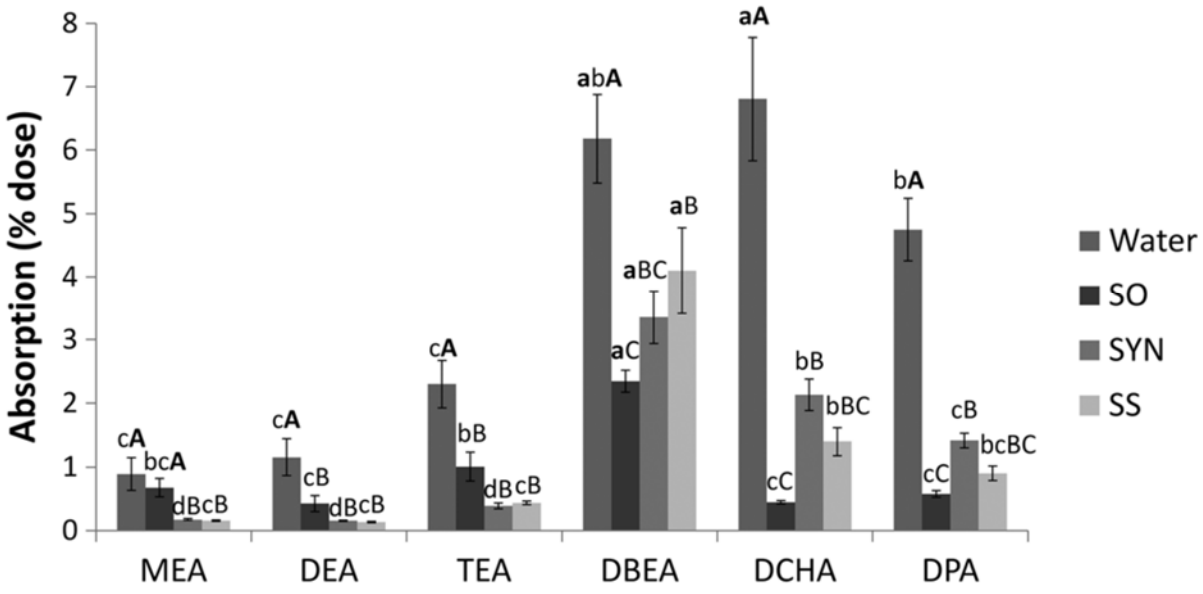


Figure 3. Mean absorption (% initial dose) of MEA, DEA, TEA, DBEA, DCHA and DPA in water, and 5% SO, 5% SYN and 5% SS type of metalworking fluids. The dose in each diffusion cell was 2500 µg. Means with the same letter are not significantly different (P > 0.05). Lower case letters indicate comparison of the means between test compounds within vehicles. Upper case letters indicate comparison of the means between vehicles within test compound. DBEA, dibutylethanolamine; DCHA, dicyclohexylamine; DEA, diethanolamine; DPA, diphenylamine; MEA, monoethanolamine; SO, soluble oil; SS, semisynthetic; SYN, synthetic; TEA, triethanolamine.

Future research will confirm these findings in an in vivo pig model along with dermatotoxicity studies. These results should help MWF industries choose safer amine-based additives for their formulations to protect the health of metalworkers.

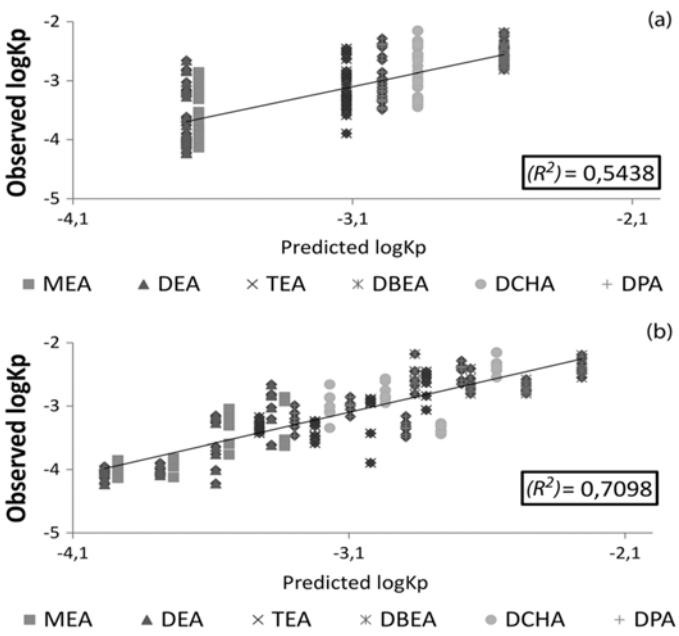


Figure 4. Test compound permeability in water and metalworking fluids using a simple Abraham multiple linear regression model (a) and adding a vehicle indicator to this model (b). Adding a coefficient for each vehicle leads to splitting the six lines (one for each tested molecule) into 24 lines (one for each vehicle for the six test compounds). The coefficient of determination (R^2) increases from 0.54 to 0.71, which indicates that predictions made using the model used to obtain (b) would be more robust.

Data obtained in this study could be pooled with previous results obtained by our laboratory (Monteiro-Rivière et al., 2006; Vijay et al., 2009), and a predictive model could be designed to estimate logKp of unknown molecules in different vehicles. With this model, industries could rapidly evaluate if molecules can easily cross the skin in different types of MWF formulations and choose the safest additives, that is, the compounds having the lowest Kp, to protect the health of metalworking employees.

Specific Aim 3*. *To quantify the effect of MWF formulations on the in vivo dermal absorption of MWF biocides.

The primary objective in this final phase of the proposal was to evaluate the robustness of our model systems to predict these formulation changes *in vivo* by topically applying selected formulations to the dorsum of weanling pigs. Our laboratory has extensively demonstrated that porcine skin is anatomically and biochemically similar to human skin, therefore any significant changes in biocide permeability would suggest that similar changes may occur in human skin following occupational exposure to MWF.

Because of concerns about "flip-flop" kinetics for several of the MWF formulations, IV studies were first conducted to assess PK parameters and the data from these IV studies will be needed to assess comparative bioavailability of the various topical MWF formulations. It was also important for us to at least capture 5-7 half-lives for the biocide to ensure that sufficient data is captured during the absorption and depletion phase in the animal. The data presented below pertain to the IV administration of OPP to weanling pigs.

Figure 1 demonstrated that OPP was eliminated very slowly over a 5 days period and data was best fitted to a 3-compartment model based on AIC comparisons and goodness of fit. The half-life of OPP was determined to be 23.7 hours, clearance was 2.8

ml/hr/kg, and volume of distribution at steady state was 150 ml/kg. This was significantly longer that earlier published data for half-life of OPP in humans. It should be noted that this earlier publication incorrectly modeled the human data.

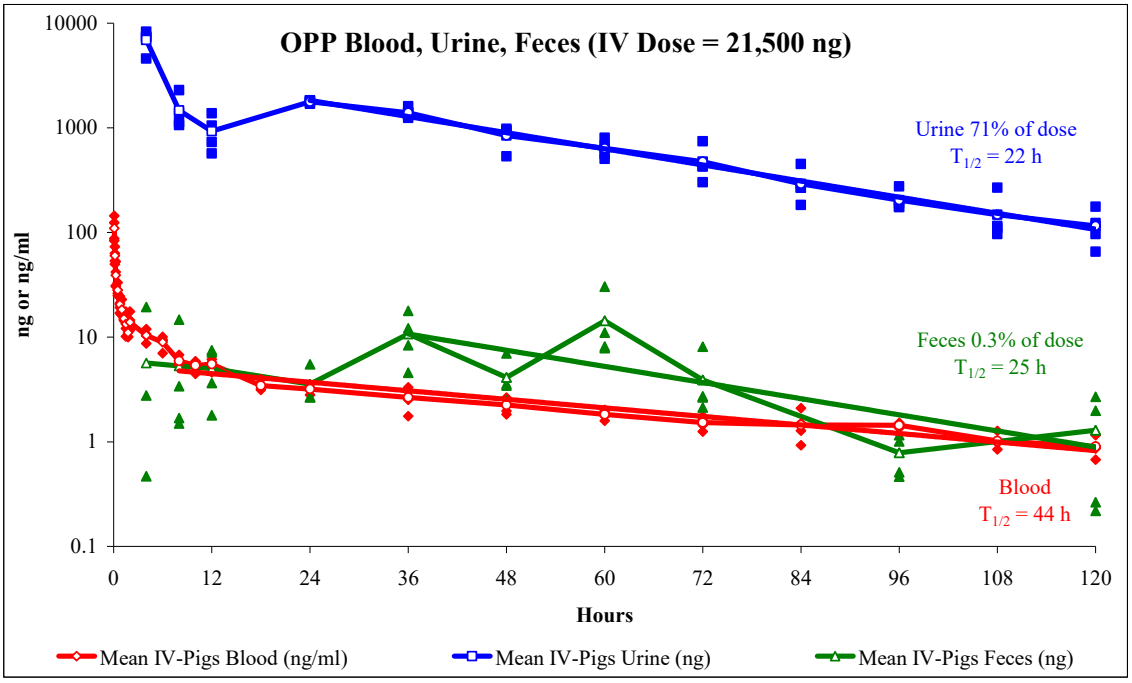


Figure 1. Blood concentration (ng/ml), and mass (ng) of urine and feces in pigs following a single **intravenous bolus** dose of o-Phenylphenol in ethanol and saline. Urine and feces are shown as total ng up to the collection time, while blood is shown as ng/ml.

Table 1 below demonstrated that 71% of the dose was eliminated in the Urine and 0.3% of the dose was eliminated in the Feces and 14.3% in the tissues 5 days after the initial IV exposure to the MWF biocide. The data was shown to best fit a 3-compartment model, and the mean plasma terminal half-life for 4 animals was 23.7 hours (CV = 23%) and mean clearance was and mean volume of distribution at steady state was 3.5 ml/hr/kg (CV = 12%) and 100 ml/kg (CV = 16%), respectively.

Table 1. Peak plasma concentrations, and total amount in urine, feces, and tissues in pigs **intravenously** dosed with o-Phenylphenol in ethanol and saline.

IV	Peak Blood (ng/ml)	Urine (Total ng)	Urine (%Dose)	Feces (Total ng)	Feces (%Dose)	Tissues (ng)	Tissues (%Dose)	Dose (ng)	Dose (ng/kg)	Recovery (% Dose)
IV_Pig1	144.1	12,870	60.9%	41	0.19%	3,427	16.2%	21,138	1,136	80.8%
IV_Pig2	87.5	14,562	76.3%	77	0.40%	2,193	11.5%	19,077	1,136	91.5%
IV_Pig3	124.2	17,665	74.5%	42	0.18%	3,902	16.5%	23,713	1,135	94.7%
IV_Pig4	83.4	15,676	70.7%	59	0.27%	2,907	13.1%	22,162	1,137	87.7%
Mean	109.8	15,193	70.6%	55	0.26%	3,107	14.3%	21,523	1,136	88.7%
SD	29.3	2,011	6.9%	17	0.10%	733	2.4%	1,944	1	6.0%
CV	27%	13%		32%		24%		9%	0%	6.8%

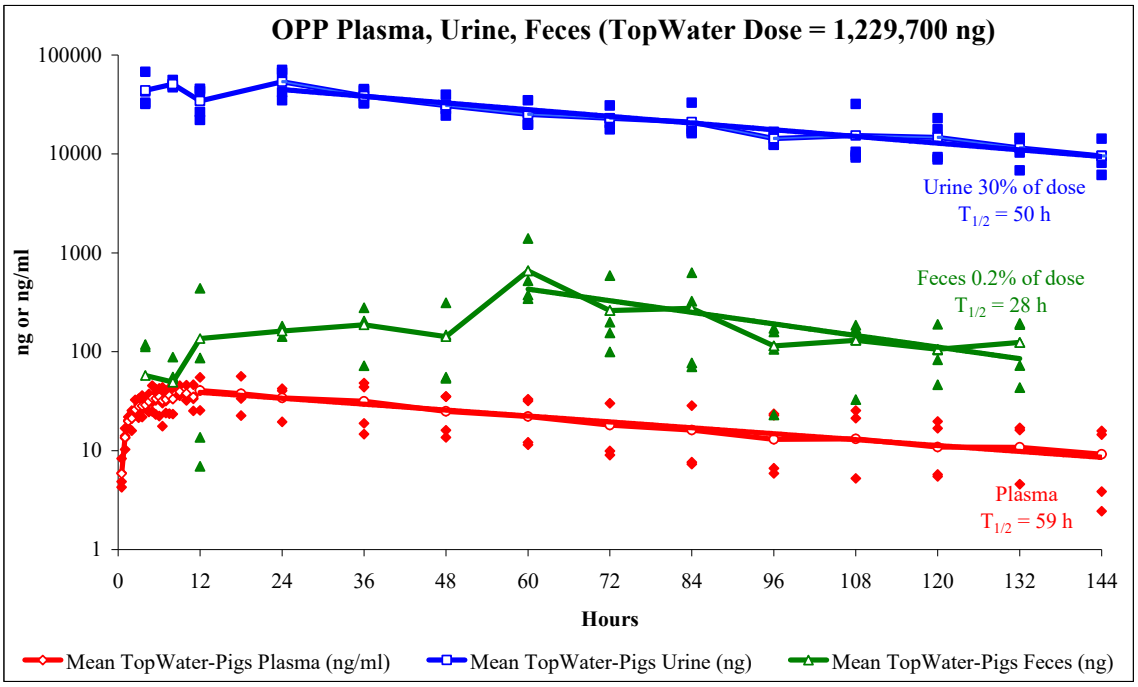


Figure 2. Plasma concentration (ng/ml), and mass (ng) of urine and feces in pigs following a single topical dose of o-Phenylphenol in 100% **Water**. Urine and feces are shown as total ng up to the collection time, while blood is shown as ng/ml.

Figure 2 depicts a very slow depletion of the drug from the blood and suggestive of steady state kinetics over the 5-day exposure. Table 2 demonstrates that 30% of the dose was eliminated in the Urine and 0.2% of the dose was eliminated in the Feces and 14% in the tissues. It can be assumed that if the dose was removed at day 5 that the dose elimination pattern seen in the IV study would also be depicted over another 5 days. Therefore, 45% of the dose was absorbed with this water only dose.

Table 2. Peak plasma concentrations, and total amount in urine, feces, and tissues in pigs topically dosed with o-Phenylphenol in 100% **Water**.

Topical Water	Peak Plasma (ng/ml)	Urine (Total ng)	Urine (%Dose)	Feces (Total ng)	Feces (%Dose)	Tissues (ng)	Tissues (%Dose)	Dose (ng)	Dose (ng/kg)	Recovery (% Dose)
Wat #5	35.9	294,298	23.9%	1,445	0.12%	179,433	14.6%	1,229,688	60,118	64.0%
Wat #6	48.2	390,934	31.8%	1,808	0.15%	177,485	14.4%	1,229,688	65,983	81.3%
Wat #7	56.3	498,571	40.5%	2,326	0.19%	170,787	13.9%	1,229,688	54,106	88.5%
Wat #8	40.9	310,692	25.3%	3,728	0.30%	174,447	14.2%	1,229,688	71,192	84.0%
Mean	45.3	373,623	30.4%	2,327	0.19%	175,538	14.3%	1,229,688	62,850	79.5%
SD	8.9	93,389	7.6%	1,002	0.08%	3,774	0.3%	0	7,379	10.7%
CV	20%	26%		46%		2%		0%	12%	13.6%

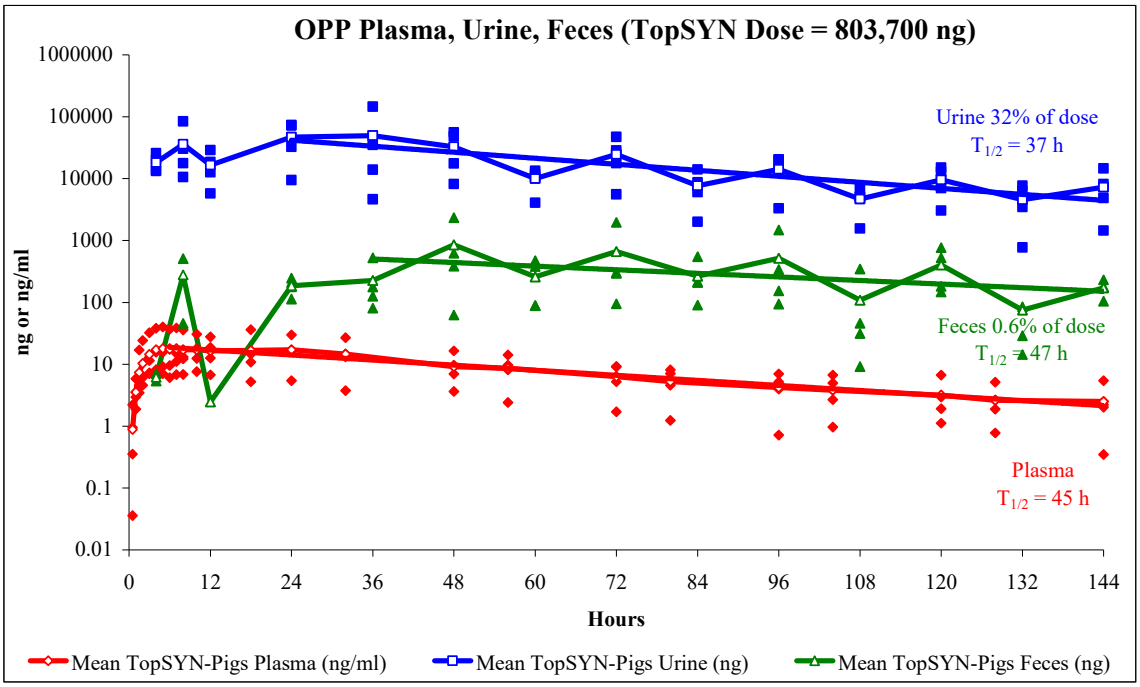


Figure 3. Plasma concentration (ng/ml), and mass (ng) of urine and feces in pigs following a single topical dose of o-Phenylphenol in 5% **Synthetic MWF** + 95% Water. Urine and feces are shown as total ng up to the collection time, while blood is shown as ng/ml.

Approximately 32% of the dose was eliminated in the Urine and 0.6% of the dose was eliminated in the Feces. This is very similar pattern to the Water dose. It should be noted that total % dose recovered in the urine, feces and tissues at 5 days of exposure for the water (45% dose) and synethetic (40% dose) MWF formulation were comparable.

Table 3. Peak plasma concentrations, and total amount in urine, feces, and tissues in pigs topically dosed with o-Phenylphenol in 5% **Synthetic MWF** + 95% Water

Topical Synthetic	Peak Plasma (ng/ml)	Urine (Total ng)	Urine (%Dose)	Feces (Total ng)	Feces (%Dose)	Tissues (ng)	Tissues (%Dose)	Dose (ng)	Dose (ng/kg)	Recovery (% Dose)
Syn #9	7.6	74,131	14.0%	1,450	0.27%	52,317	9.9%	528,526	29,069	38.6%
Syn #10	16.5	187,160	33.5%	8,564	1.53%	49,931	8.9%	558,369	36,130	91.5%
Syn #11	18.8	446,871	40.3%	2,922	0.26%	52,932	4.8%	1,107,518	56,664	97.3%
Syn #12	40.2	421,006	41.3%	2,376	0.23%	59,619	5.8%	1,020,328	52,203	105.6%
Average	20.8	282,292	32.3%	3,828	0.58%	53,700	7.4%	803,685	43,516	83.2%
SD	13.8	181,392	12.7%	3,215	0.64%	4,153	2.4%	302,843	13,059	30.3%
CV	79%	80%		105%		8%		40%	31%	39.1%

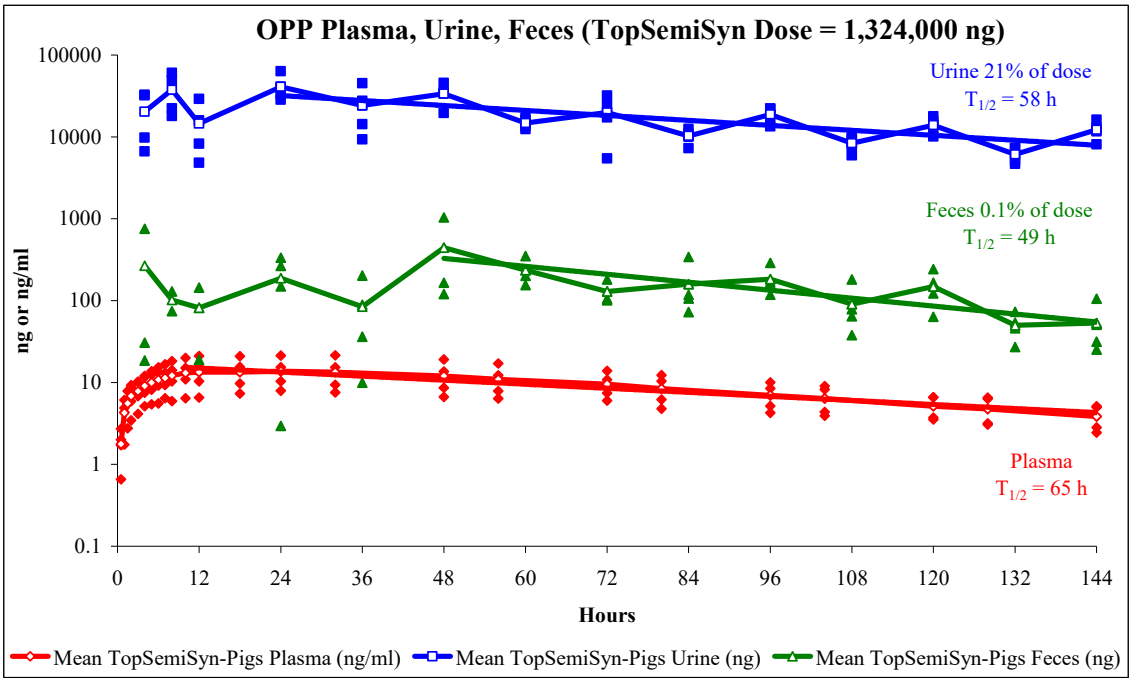


Figure 4. Plasma concentration (ng/ml), and mass (ng) of urine and feces in pigs following a single topical dose of o-Phenylphenol in 5% **Semi-synthetic MWF** + 95% Water. Urine and feces are shown as total ng up to the collection time, while blood is shown as ng/ml.

Approximately 21% of the dose was eliminated in the Urine and 0.1% of the dose was eliminated in the Feces. Compared the previous 2 more aqueous formulations (Water and Synthetic MWF) (40-45% dose), there was significantly less skin absorption from Semi-synthetic MWF compared with based on biocide concentrations recovered in urine, feces and tissues; that is, approximately 27% of the dose.

Table 4. Peak plasma concentrations, and total amount in urine, feces, and tissues in pigs topically dosed with o-Phenylphenol in 5% **Semi-synthetic MWF** + 95% Water

Topical Semi-Synthetic	Peak Plasma (ng/ml)	Urine (Total ng)	Urine (%Dose)	Feces (Total ng)	Feces (%Dose)	Tissues (ng)	Tissues (%Dose)	Dose (ng)	Dose (ng/kg)	Recovery (% Dose)
SS #13	21.4	380,100	28.7%	1,228	0.09%	90,319	6.8%	1,324,492	44,150	77.7%
SS #14	7.9	173,470	13.1%	1,802	0.14%	80,412	6.1%	1,325,034	43,509	79.1%
SS #15	15.7	338,584	25.6%	3,249	0.25%	84,776	6.4%	1,325,034	43,509	79.9%
SS #16	10.9	211,262	16.0%	1,229	0.09%	73,486	5.6%	1,321,784	47,671	56.1%
Mean	14.0	275,854	20.8%	1,877	0.14%	82,248	6.2%	1,324,086	44,709	73.2%
SD	5.9	99,091	7.5%	954	0.07%	7,111	0.5%	1,555	1,997	11.4%
CV	45%	38%		55%		9%		0%	4%	15.8%

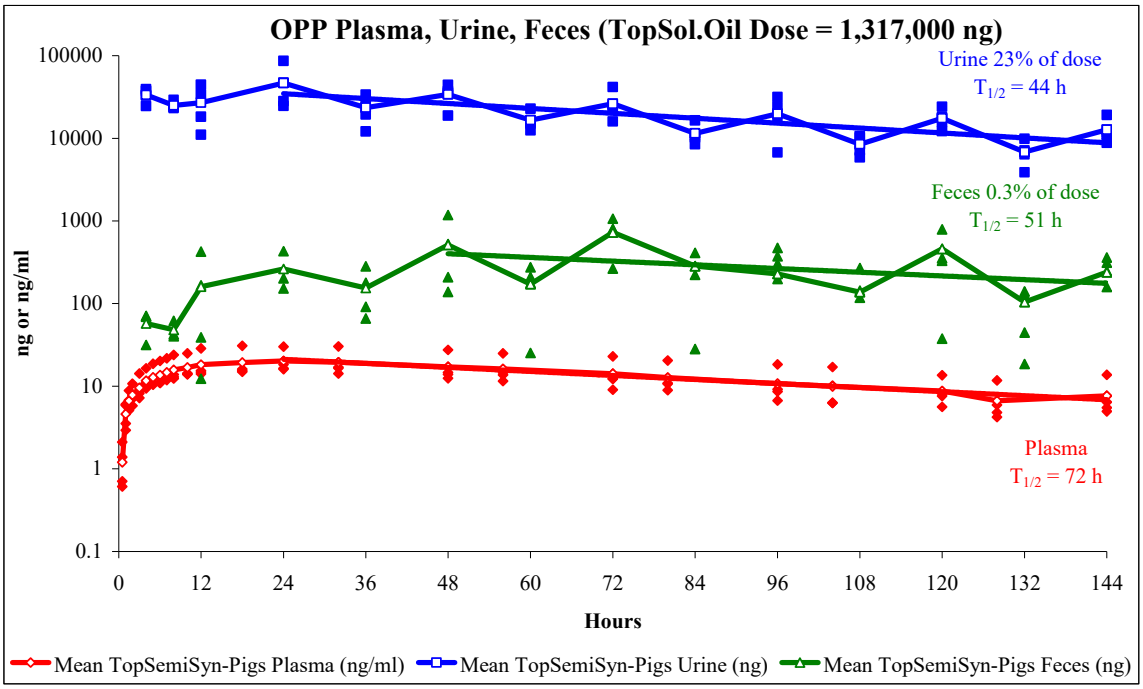


Figure 5. Plasma concentration (ng/ml), and mass (ng) of urine and feces in pigs following a single topical dose of o-Phenylphenol in 5% **Soluble Oil MWF** + 95% Water. Urine and feces are shown as total ng up to the collection time, while blood is shown as ng/ml.

Approximately 23% of the dose was eliminated in the Urine and 0.3% of the dose was eliminated in the Feces. As observed with the Semi-synthetic MWF, less of the biocide was apparently systemically absorbed with soluble oil MWF when compared with synthetic MWF. These observations are based on percentage dose determined in the urine, feces and tissues (32% dose) over a 5-day skin exposure to biocide in the MWF formulation.

Table 5. Peak plasma concentrations, and total amount in urine, feces, and tissues in pigs topically dosed with o-Phenylphenol in 5% **Soluble Oil MWF** + 95% Water

Topical Soluble Oil	Peak Plasma (ng/ml)	Urine (Total ng)	Urine (%Dose)	Feces (Total ng)	Feces (%Dose)	Tissues (ng)	Tissues (%Dose)	Dose (ng)	Dose (ng/kg)	Recovery (% Dose)
SO#17	16.4	321,912	24.4%	3,957	0.30%	90,424	6.9%	1,317,452	54,687	83.0%
SO#18	30.9	442,439	33.6%	3,233	0.25%	123,201	9.4%	1,316,369	56,785	82.4%
SO#19	17.0	191,240	14.5%	2,800	0.21%	84,000	6.4%	1,317,452	54,687	74.5%
SO#20	18.3	281,063	21.3%	3,495	0.27%	97,153	7.4%	1,318,535	52,741	71.0%
Mean	20.7	309,164	23.5%	3,371	0.26%	98,694	7.5%	1,317,452	54,725	77.7%
SD	6.8	104,276	7.9%	485	0.04%	17,198	1.3%	884	1,651	5.9%
CV	34%	35%		14%		18%		0%	3%	7.6%

CONCLUSIONS

We used a single membrane-coated fiber (MCF), the PDMS fiber, that most represents skin and to assess a more complex experimental conditions which is more representative of industry such as range of solute chemical concentrations (n = 6), a range of MWF concentrations (n = 3), and five (n = 5) different formulations for as many as 34 different chemicals. This represents 90 potential exposure scenarios for each of these chemicals which is beyond the original proposal; however, we limited these exposures to a single MCF. We extended the one-factor expanded Abraham model proposed in Xu et al. 2013 to three factors, that not only **accounts for complex experimental conditions but also improved the ability to conduct the rigorous hypothesis testing.** Data from these MCF studies demonstrated that the expanded Abraham model not only has a better model fit and predictive power, but also the power of testing various scientific hypothesis. **This approach was able to identify significant MWF formulation effects and concentration effects for limited number of solutes; the former was anticipated but we did not anticipate the latter.** That is**,** for many of the exposure conditions we investigated, the analyte concentration effect was signficant and the partion theory was violated. Our data also suggest that **increasing the MWF tank-side concentration decreases the absorption of highly lipophilic compounds** and the more concentrated the MWF the more lipophilic the environment and therefore the more likely for those chemical solutes with high Ko/w to remain in the MWF and not absorbed. However, it should be noted that skin irritation studies should be run to determine if constituents in the MWF increase irritation with increased concentrations.

The work in the next phase of this project resulted in development of a comprehensive procedure to select training sets from a large candidate set of 4534 solutes. A newly proposed 'Baynes' rule', which is a modification of Lipinski's 'rule of five', was used to screen out solutes that were not qualified for the study. U-optimality was used as the selection criterion. A principal component analysis showed that the selected training set was representative of the chemical space. Gas chromatograph amenability was verified. A model built using the training set was shown to have greater predictive power than a model built using a previous dataset developed in our laboratory.

Our QSAR approach with in vitro skin demonstrated that skin permeability was shown to have the rank order: water > synthetic > semisynthetic > soluble oil. Our expanded QSAR model improved both the model fit and the predictive power when compared to the simple model in the previous grant period. **More importantly, our data analysis implies that skin permeability in water or synthetic MWF is likely to be three (3) times greater than the permeability in soluble oil**.

Furthermore, our in vitro studies demonstrated the influence of metal working fluids (MWF) on the absorption of various biocides with a focus on phenolics and amines. This research was the first to demonstrate the dermal absorption of dicyclohexylamine (DCHA) and other related amines and the effects of MWF formulations on their skin absorption. DCHA is important as some in the industry use this amine amongst others as a biocide although it is registered only as a corrosive inhibitor but it also has biocidal activities. We discovered that soluble oil tended to increase permeability of hydrophilic amines and the reverse effects on lipophilic amines such as DCHA.

The in vivo studies were focused on the intravenous (IV) and transdermal kinetics of o-phenylphenol (OPP) because this biocide represented significant formulation effects from the previous *in vitro* skin permeability studies. The IV studies were the first to determine the kinetics of OPP in pigs and the transdermal studies were the first ever in vivo studies to assess biocide absorption across several (4) MWF formulations. Our studies demonstrated that the OPP has a long half-life (23 hrs) which is similar to earlier reported human studies but was extended with topical exposure to MWF formulations especially the SO formulation compared to the SYN formulation. The data also suggest that over a 5 day exposure to either of the four (4) MWFs, that skin absorption was greater in aqueous MWFs (e.g., SYN formulations) compared to the SO formulation. Future analysis will focus on dissecting out other formulation differences. In conclusion, these in vivo studies demonstrated several of the trends observed in the MCF and *in vitro* studies.

In conclusion, this project utilized three (3) model systems to assess skin absorption of chemicals such as MWF biocides in various MWFs used in the industry. The MCF model was able to screen for formulation, formulation concentration, and chemical concentration effects albeit with one MCF. The *in vitro* studies allowed for the development of an expanded QSAR model for targeted MWF formulations and we are able to validate several of these observation using an *in vivo* model for a popular phenolic biocide, OPP, still used by some in the metalworking industry. However, these observations were based on a defined chemical space, but the developed models allows for future hypothesis testing. This becomes important when considering whether these findings can be applied to assessing adverse effects of these chemicals/biocides when the skin of workers are exposed to these various MWF formulations. These studies did not assess depot formation in the skin which may be more likely for some chemicals than others that are absorbed into the blood stream. These chemicals should be better identified if there is concern for local irritant effects rather than systemic effects.

PUBLICATIONS GENERATED FROM THIS GRANT:

This new information has been presented at relevant occupational health and toxicology conferences and publications in peer-review publications. This has been documented in the following 10 publications (peer-review or published abstracts) below for the last 4 years with 3 of these manuscripts at different stages of preparation:

Peer-Reviewed Publications.

Nixon, E, Brooks, J.D., Routh, P., and Baynes R.E. (2016). Metalworking fluid formulation effects on the absorption of the biocide, *ortho*-phenylphenol: an *in vitro – in vivo* comparison in porcine skin. *J. Appl. Toxicol*. (*in preparation*).

Nixon, E, Brooks, J.D., Routh, P., and Baynes R.E. (2016). Intravenous pharmacokinetics of *ortho*-phenylphenol in pigs. *Hum. Exp. Toxicol*. (*in preparation*).

Xu G, Hughes-Oliver JM, Brooks JD, Baynes RE. (2016). Use of membrane coated fibers (MCF) to screen and assess complex mixture interactions relevant to metalworking fluids. *SAR QSAR Environ Res. (in preparation).*

Roux, LN., Brooks, JD., Yeatts, JL., and Baynes, R.E. (2015). Skin absorption of six performance amines used in metalworking fluids. *J. Appl. Toxicol*. 35(5):520-528.

Xu G, Hughes-Oliver JM, Brooks JD, Baynes RE. (2013). Predicting Skin Permeability from Complex Chemical Mixtures: Incorporation of an Expanded QSAR Model. SAR QSAR Environ Res. DOI: 10.1080/1062936X.2013.792875

Xu G, Hughes-Oliver JM, Brooks JD, Yeatts JL, Baynes RE. (2013). Selection of appropriate training and validation set chemicals for modeling dermal permeability by U-optimal design. SAR QSAR Environ Res., 24(2):135-156.

Poster Presentations & Abstracts

Roux, LN., Brooks, JD., Yeatts, JL., and Baynes, R.E. (2014). Skin absorption of performance amines used in metalworking fluids *Toxicologist.* Vol 138 (1); PS 2175. Society of Toxicology 53rd Annual Meeting, Phoenix, AZ. March 10th –14th, 2014.

Linthicum, A., Inman, A., Monteiro-Riviere, NA., and Baynes, R.E. (2013). Cytotoxic Effects of Dicyclohexylamine and Three Metalworking Fluids on Human Epidermal Keratinocytes. Toxicologist. Society of Toxicology 52st Annual Meeting, San Antonio, TX March 10th –14th, 2013.

Linthicum, A., Yeatts, L., Brooks, JB, Koivisto, E., and Baynes, R.E. (2012). In Vitro Dermal Absorption of a Metalworking Fluid Additive: Dicyclohexylamine (DCHA). *Toxicologist.* Society of Toxicology 51st Annual Meeting, San Francisco, CA March 11th – 15th, 2012.

Baynes RE (2012). Dermal Absorption Assessment of Metal Working Fluid (MWF) Formulations. X2012 - 7th International Conference on the Science of Exposure Assessment Edinburgh, Scotland, July 1-4th, 2012.