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In vitro Microdialysis Sampling Collection of Volatile Organic Compounds (VOC's), Dodecafluoropentane (DDFP) and Isoflurane

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In vitro Microdialysis Sampling Collection of Volatile Organic Compounds (VOC's), Dodecafluoropentane (DDFP) and Isoflurane

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By

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Abstract

Death by stroke occurs every four minutes to human beings. Strokes cause necrosis within the tissue of the brain due to deprivation of oxygen. Perfluorocarbons have the ability to transport oxygen to tissue and in return decrease cell death. Dodecafluoropentane (DDFP) is a volatile fluorocarbon and collection *in vivo* can be a challenge since this compound evaporates at room temperature. There is currently not an efficient collection method *in vivo* for compounds that are volatile. Without a method to collect DDFP it is impossible to be approved for clinical use since exact concentrations of the drug within the body will be unknown. The current *in vitro* work demonstrates that microdialysis can collect volatile organic compounds, isoflurane (a standard inhalation anesthetic) and DDFP. Different perfusion fluids and flow rates were tested for optimal analyte collection through the microdialysis membrane. Instead of utilizing an aqueous perfusion fluid safflower oil and air was passed through the microdialysis probe. The perfusion fluid and flow rate of choice for isoflurane sampling was safflower oil at 0.5 µL/min, respectively. For DDFP there was no significance in flux between flow rates and air was a more suitable perfusate. Since oil was a potential candidate as a perfusion fluid through the microdialysis probe, the oil/air partition coefficients ($K_{Oil/Air}$) were calculated. $K_{Oil/Air}$ was determined because the analyte in the air phase will partition into the oil phase that is being perfused through the microdialysis probe. The average calculated $K_{Oil/Air}$ for Isoflurane and DDFP was 10.62 and 0.53, respectively. These values lead to isoflurane partitioning more into oil vs. air and DDFP partitioning more into air vs. oil. The new analytical method described here shows that VOC's can be collected with microdialysis sampling technique and thus serves as a starting point for *in vivo* collections has been found.

Acknowledgments

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I would like to thank my lab mates for their support throughout the years and for the wonderful memories they have given me.

Dedication

I would like to dedicate my thesis to my grandparents Issac Elbaz and Esther Elbaz. Je vous remercie de votre confiance et de votre soutien tout au long de ma vie. To my father and mother, Lonnie McKinney and Jacqueline McKinney for the continuous sacrifice and support. To my sister Alexandra Hernandez for her constant encouragement and friendship. To my fur babies, Bonaparte and Pepper, for their unconditional love.

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Abbreviations

AHA	American Heart Association
bp	Boiling Point
DDFP	Dodecafluoropentane
DDFPe	Dodecafluoropentane Emulsions
ECD	Electron Capture Detector
ECF	Extracellular Fluid
EI	Electron Impact
FEP	Fluorinated Ethylene Propylene
HS-GC-FID	Headspace Gas Chromatography Flame Ionization Detector
HS-GC-MS	Headspace Gas Chromatography Mass Spectrometry
INJ	Injector Port
K	Partition Coefficient
TCD	Thermal Conductivity Detector
TIMI	Thrombolysis in Myocardial Infarction
t_R	Retention Time
TTC	Triphenyltetrazolium Chloride
VOC's	Volatile Organic Compounds
WHO	World Health Organization

Introduction

Significance

Dodecaflouropentane (DDFP) has been shown to be an excellent candidate for oxygen transportation by decreasing the infarct volume in rabbit brain after stroke.[1, 2] DDFP is a volatile organic compound (VOC) that is hydrophobic with low boiling point (bp) of 29˚C. With such a low bp this compound evaporates at room temperature making it challenging to analyze. There is currently not a sufficient method to collect volatile organic compounds (VOC's) *in vivo*. By using the microdialysis sampling technique, a real time concentration profile may be determined for DDFP and other VOC's allowing them to proceed to clinical trials. Before *in vivo* sampling can begin *in vitro* microdialysis experiments need to be conducted to prove that DDFP and other VOC's can be collected with this microdialysis sampling setup.

Background

There are numerous conditions that impair oxygen transportation to tissue. The deprivation of oxygen eventually leads to tissue damage and or death. Some of these ailments are caused by trauma and natural diseases. A disease that has harshly impacted the world is stroke. This disease has predominantly two forms - ischemic and hemorrhagic stroke. The American Heart Association (AHA) defines a hemorrhagic stroke as when a blood vessel's stability diminishes and causes a fissure in the blood vessel.[3] The AHA defines an ischemic stroke as a blood vessel having a blockage.[4] This vessel blockage does not allow adequate provisions of blood to the brain. The World Health Organization (WHO) reported that stroke and ischemic heart disease was the number one leading cause of death in women in 2011 and it has been ranked as the number two cause of death in the world as of 2012.[5, 6] In the United States stroke is ranked number four in the leading causes of death.[1]

Dodecafluoropentane Properties and Benefits

Dodecafluoropentane has the ability to transport and deliver oxygen. [7] This fluorocarbon is also hydrophobic. This allows for the fluorocarbon to pass primarily into tissues.[7] DDFP has an average half-life in humans of 2.2±1.2 minutes in blood and a 98±19% recovery at 2 hours from the injection into the peripheral vein.[8] Once DDFP has been injected intravenously it is exhaled from the lungs.[9] The molecule also has a linear geometry allowing it to have a higher affinity for O_2.[10] Another interesting characteristic of DDFP is that it can exist as an emulsion. These dodecafluoropentane emulsions (DDFPe) stabilize the compound and are much smaller than erythrocytes.[7] The size of these emulsions range from 250 – 300 nanometers.[1] The DDFPe have been shown to be effective oxygen carriers with rabbit stroke models.[1, 2] The model demonstrated that there was a reduction in the median infarct volume within the brain as shown in Figure 1.[1]

Stroke is not the only ailment that DDFP can aid. DDFP has been tested for the radiosensitization of Morris 7777 hepatoma. There was no significant sensitization with just DDFP alone but when combined with carbogen the tumor was no longer impervious to sensitization.[11] DDFP has also been recognized as a relevant ultrasound contrast agent.[12] In an *in vitro* study of DDFP as a contrast agent showed that 98±4% of the clot was almost entirely eradicated.[13] *In vivo* studies with different thrombolysis in myocardial infarction (TIMI) flow grades also exhibited a considerable amount of recanalization when DDFP was combined with ultrasound.[13] This fluorocarbon may also play a role in organ preservation and carbon monoxide poisoning in the future.[7, 14]

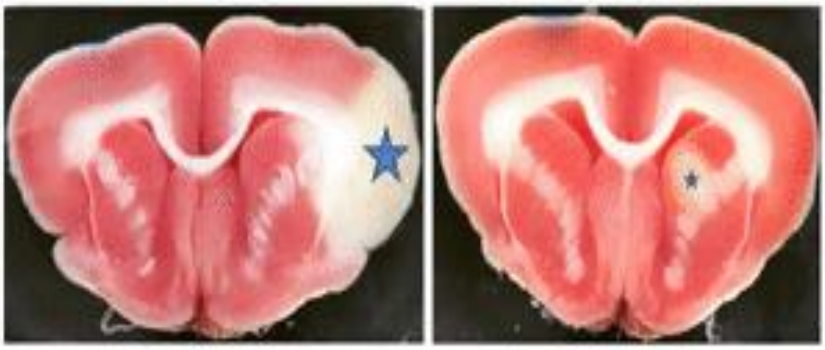


Fig. 1 demonstrates the infarct volume with triphenyltetrazolium chloride (TTC) staining of rabbit brain slices. The control is shown on the left and the treated (DDFPe) is on the right. The control shows a significant amount of tissue death (3.9%) whereas treated shows very little (0.86%). Springer and Molecular Neurobiology, 48, 2013, 364, Progress in Dodecafluoropentane Emulsion as a Neuroprotective Agent in a Rabbit Stroke Model, Woods et. al., Figure 1, 2013, is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

In order for this compound to proceed into clinical trials a real time concentration profile needs to be established within the brain tissue. Current research has only allowed for recovery determination from intravenous blood samples and whole tissue sample analysis. These methods lack the ability to produce a real time concentration profile for the brain. A sampling technique that will allow for a real time concentration profile to be established within the brain is microdialysis sampling. Since microdialysis sampling will be utilized for collection of the analyte there are parameters that need consideration. The first issue that will need to be addressed is the partition coefficient (K). The analyte will partition between the matrix and the perfusion fluid. To quantitatively determine the concentration of DDFP delivered to the brain via intravenous injection the, perfusion fluid/extracellular fluid (ECF) partition coefficients needs to be calculated. Another aspect to consider when using microdialysis is the perfusion fluid. The perfusion fluid or the perfusate is a fluid that is passed through the probe to collect the desired analyte. The analyte will not have the same K value with different perfusion fluids and different ECF's. When choosing a perfusion fluid there must be thought based upon what tissue the sampling is occurring at as well as the properties of the analyte. For in vitro work with DDFP different perfusion fluids (water, safflower oil, and air) were chosen solely on the analytes properties. These perfusion fluids were tested to see if DDFP could be collected in dialysate. Water was tested and DDFP was not detected. This was expected since this compound is hydrophobic. The next perfusion fluid tested was safflower oil. Safflower oil was tested because it is a lipid and DDFP is lipophilic. The outcome was that safflower oil was able to collect DDFP. The last perfusate tested was air. Air was chosen under the assumption that DDFP would have a higher concentration in the gas phase. Air also had the ability to collect

DDFP. Once the perfusion fluid was chosen the partition coefficient between perfusate/air will be determined and the recovery of DDFP *in vitro* can be quantified via microdialysis.

Microdialysis

Microdialysis is a minimally-invasive diffusion based sampling technique that allows for the constant collection of unbound molecules in various matrices (Figure 2). The ability for the continuous collection of a specific analyte is caused from the concentration gradient between the perfusate and the fluid at the sampling site.[15] The perfusion fluid is pushed through the inlet of the probe by a syringe that is connected to a pump that controls varying flow rates (0.5-2.0 μL/min)[16]. Next the perfusate travels through the semi-permeable membrane of the probe. The length of membrane is 4-10 mm with an outer diameter of 200-500 μm. [17] The semi-permeable membrane allows molecules from the fluid at the sampling site to diffuse to and from the perfusate.[18] The analyte will then travel from an area of high concentration to low concentration.[19] . The semi-permeable membrane also comes with a determined molecular weight cutoff (MWCO) normally 20 or 100 kDa. The MWCO allows for the ability for only certain molecules with that set molecular weight to pass freely across the semi-permeable membrane. After the molecules have traversed the membrane the perfusion fluid transports the analyte to a vial where the dialysate is collected.[20]

The molecules collected from microdialysis sampling technique are most commonly small endogenous and hydrophilic molecules such as dopamine and glutamate.[21] Microdialysis has been proven to not only collect endogenous molecules but to also collect exogenous molecules such as drugs that are administered into the body.[21] Another benefit of microdialysis is that it can be implanted at various sites for instance, one can sample to a particular site in the brain or one can choose to sample in a section of subcutaneous tissue.[22]

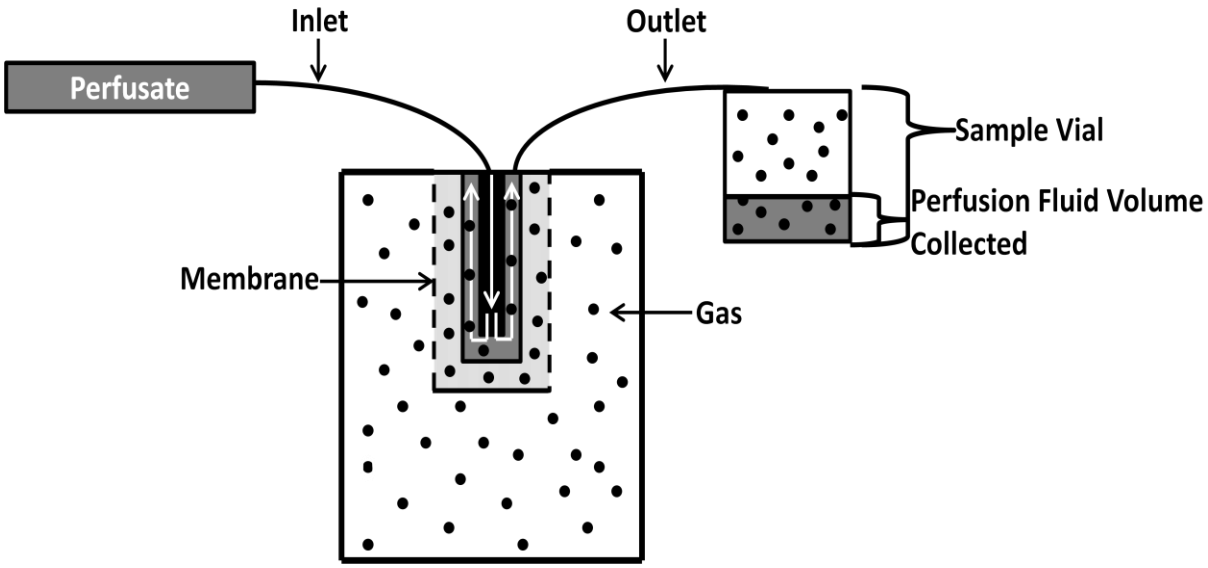


Fig. 2 A diagram of microdialysis sampling technique for VOC's showing the semipermeable membrane allowing free molecules to diffuse in and out and travel from high concentration to low concentration.

Microdialysis is a valuable sampling technique that allows for the collection of analyte at a particular site while the animal is awake and freely moving.[15]

Although microdialysis excels at collecting low molecular weight hydrophilic molecules it is not well known for collecting VOC's. It has been shown that microdialysis can collect VOC's but with low recoveries.[23] In the Jones et al. paper, mentioned previously, the recoveries obtained from this method for toluene and ethanol were 1.28% and 0.054% respectively. [23] Jones directly connected the inlet of a microdialysis probe to a Helium tank and the outlet of the probe was connected to a GC-FID (Gas Chromatography-Flame Ionization Detector) .[23] Helium was Jones choice of perfusion fluid .[23] Since the compounds for my research are both hydrophobic the common aqueous perfusion fluid will repel both DDFP and isoflurane and therefore cannot be used. Testing different perfusion fluids and different flow rates with DDFP and isoflurane are a necessity to obtain best recoveries and flux data (the amount of mass per unit area collected over time). The flux of these molecules was obtained instead of recovery since different flow rates were tested.

Figure 3 demonstrates how different perfusion fluids will affect mass transport with the specific VOC's presented in this research. Utilizing water as a perfusate, a polar compound, will cause non polar molecules to be repelled. When working with hydrophobic molecules oil is an option because of DDFP and isoflurane are lipophilic. When testing different perfusion fluids the partition coefficient will need to be calculated because there is a potential that there will be higher concentrations in the non-water phases as shown in Figure 4.

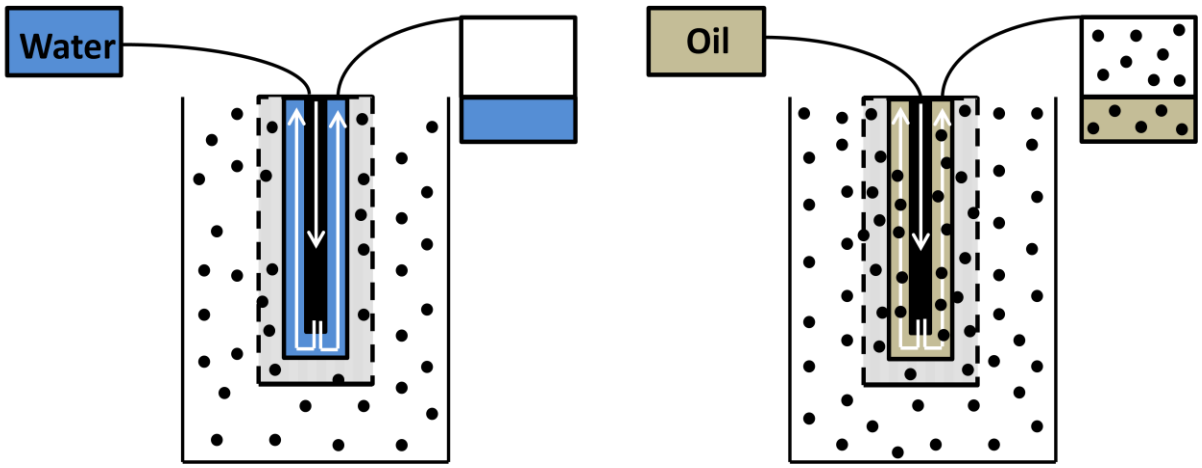


Fig. 3 Displays diffusion/mass transport of molecules with different perfusion fluids.

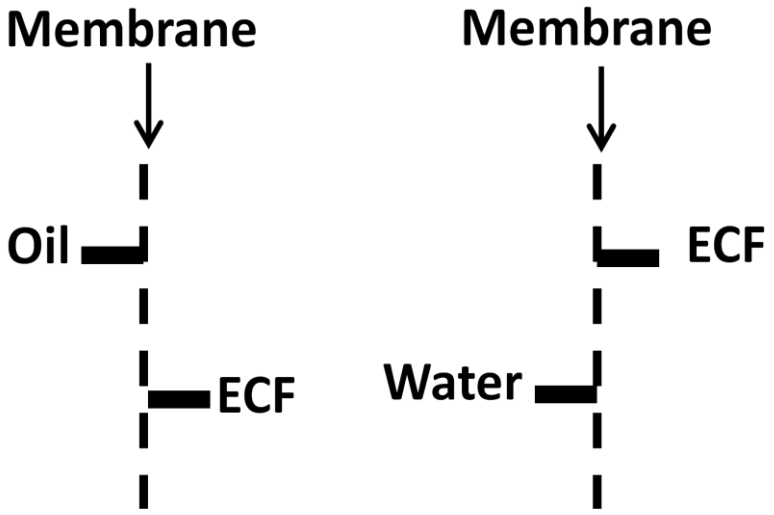


Fig. 4 When using oil as a perfusion fluid the partition coefficient could be greater than one causing an increase in the concentration of the analyte.

Partition Coefficient

The partition coefficient is the distribution of an analyte between two immiscible phases as portrayed in Figure 5. There are a variety of immiscible phases to choose from when determining the partition coefficient. A few common phases are octanol and water, oil and water, tissue and blood, and blood and water. The phases chosen for this research were safflower oil and air. The phases were chosen because of both compounds being lipophilic and volatile. This value can be established by a general partition coefficient Eq. (1).[24]

$$K = \frac{[C_s]_2}{[C_s]_1} \qquad (1)$$

K is the partition coefficient, C_{s2} is concentration of solute in phase 2 and C_{s1} represents the concentration of solute in phase 1. Since DDFP is an extremely volatile compound the detection method of choice is headspace gas chromatography with a flame ionization detector (HS-GC-FID).[25] Headspace sampling is a very common sampling technique for extremely volatile compounds and is applicable in many fields. With headspace sampling the gas phase above the sample is analyzed (Figure 6).[25]

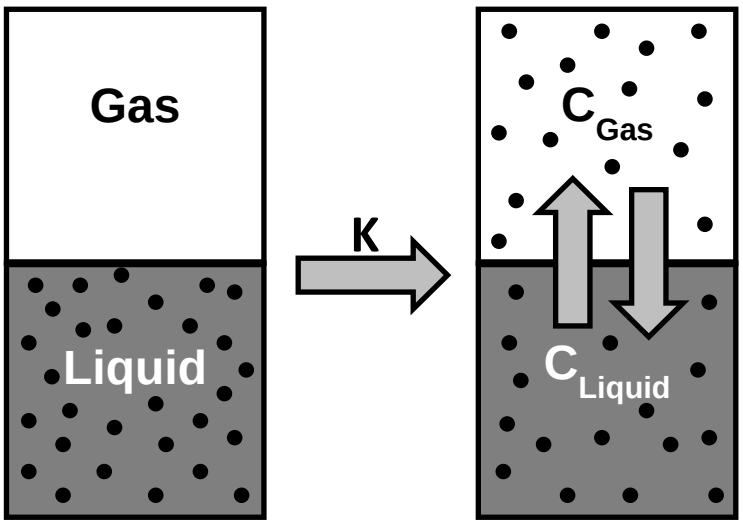


Fig. 5 An illustration of the partition coefficient.

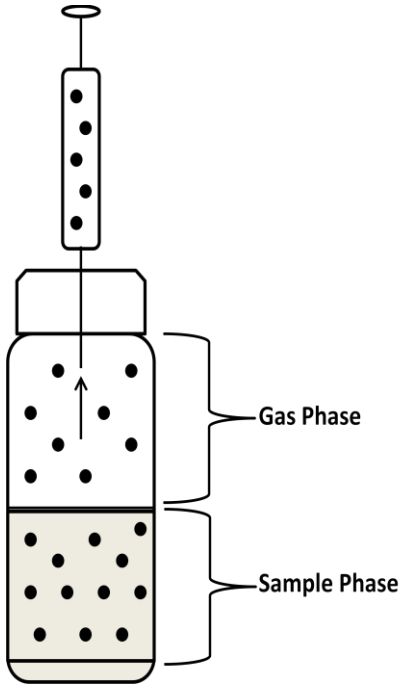


Fig. 6 A representation of headspace sampling. A syringe is inserted into the septum of the headspace vial and the gas phase is pulled out and injected into the injector port of a GC.

When analyzing the partition coefficients of volatile compounds with (HS-GC-FID) other equations need to be incorporated. In Eq. (2) below is the relationship between the partition coefficient and HS-GC.[24]

$$A\alpha C_G = \frac{C_o}{K+\beta} \qquad (2)$$

The peak area is denoted by A which is proportional (α) to the concentration of the gas phase (C_G), C_o is for the initial concentration, and β is for the phase ratio. The phase ratio can be calculated from the volume of the gas phase divided by volume of the sample phase or liquid phase. From this equation the partition coefficient can be solved. Now Eq. 1 can be rearranged to form Eq. 3 which will allow for the determination of the concentration of solute in phase 2, since the concentration in solute 1 is the same as C_G.

$$K \times C_G = C_s \qquad (3)$$

C_s represents the concentration in the sample phase.

The $K_{Oil/ECF}$ of DDFP needs to be measured because there is no way to even be semi-quantitative when determining the concentration of the analyte in the ECF phase. The $K_{Oil/Air}$ can be calculated separately from the $K_{ECF/Air}$ to give the final $K_{Oil/ECF}$. For DDFP, no K values have been reported. This value can show the distribution of DDFP throughout the body and it can show whether or not the molecule is lipophobic or lipophilic.[26, 27]

Gas Chromatography

Gas chromatography is a technique used to separate compounds by means of an interaction with a stationary phase while being carried through a column by a gaseous mobile phase. The sample must be volatilized before entering the column this can be done either from the compound already being completely volatile or by increasing the injector port temperature at or above the bp of the sample.[25] If the sample is nonvolatile there are processes that the sample can undergo to make it volatile, such processes are derivitization or pyrolysis.[28] Once the sample is injected, it will go through the column where the analytes will start to interact with the column's stationary phase depending on the properties of the analytes and the column. Some properties that account for the interaction with the stationary phase of the column are solubility and polarity. [29] These interactions cause a specific retention time (t_R) for analytes. The t_R is the time the analytes spend from injection to detection. Once the compounds leaves the column they are transported by the carrier gas directly to the detector. When the analytes reach the detector a signal output is produced in the form of peaks. The peaks must have good resolution. Resolution is the separation of peaks.[30] A good resolution would be 1.0 and the best resolution would be 1.5. Anything below 1.0 would have a poor resolution and may not be distinguishable from other peaks.[30] A GC can be coupled with many detectors such as a thermal conductivity detector (TCD), an FID and electron capture detector (ECD).[28] In Figure 7 is a schematic of a typical GC coupled with and FID.

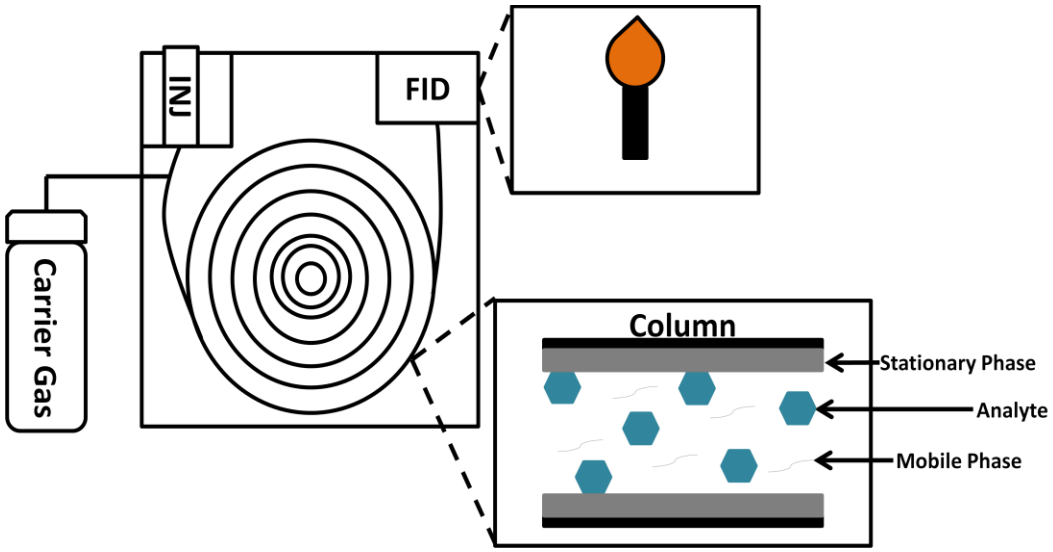


Fig. 7 A GC coupled to an FID. The sample is injected into the injector port (INJ). Once in the INJ the carrier gas carries the analytes through the column where the analyte then interacts with the stationary phase. Next the analyte is carried to the detector.

Methods

Materials

Perfluroro-n-pentane (DDFP), (99%, DDFP, CAS no. 678-26-2) was purchased from Exfluor Research Corporation, Round Rock, TX. Isoflurane was obtained from Henry Schein Animal Health, Dublin, OH. LouAna safflower oil (100%) was acquired from Ventura Foods, LLC Brea, Ca. Clear headspace vials (20.0 mL) and PTFE blue silicone magnetic screw caps (18.0 mm) were purchased from Thermo Fisher Scientific, Waltham, Massachusetts. Gastight syringes (10.0 μL and 100.0 μL) purchased from Hamilton Company, Reno, Nevada. Microdialysis probes (CMA 20 Polyethersulfone (PES) membrane, membrane length 10 mm, 100kDa molecular weight cut-off MWCO) purchased from CMA Microdialysis, Harvard Apparatus, Holliston, MA. Syringe pumps (BASi Bee) were purchased from BASI, West Lafayette, IN.

Detection of DDFP after Collection with Microdialysis

For the creation of the DDFP samples nine 20.0 mL headspace vials were capped and then crimped. The nine vials were then taken to the cold room (2.8˚C). Once in the cold room DDFP was injected into the headspace vials using a 10.0 μL gas tight glass syringe through the septum. The first sets of triplicates were each injected with 2.5 μL of DDFP. The second sets of triplicates were injected with 3.5 μL of DDFP. The last set of triplicates was injected with 4.5 μL of DDFP. Next a microdialysis setup was constructed for the detection of DDFP. The setup consisted of three pumps with three 1000.0 μL syringes as shown in Figure 8. The syringes were filled with the perfusion fluid (safflower oil, LouAna safflower oil (100%) Ventura Foods, LLC Brea, Ca). Three CMA 20 microdialysis probes with a molecular weight cutoff (MWCO) of 100 kDa were used. Each microdialysis probe was inserted into an individual headspace vial by

means of an introducer. An introducer is displayed in Figure 9. After all three probes were inserted into the headspace vial the inlet of the microdialysis probe was connected to the needle of a 1.0 mL syringe with a connector.

Next a small piece of fluorinated ethylene propylene (FEP) tubing was inserted into three different headspace vials by threading the tubing through the backside of a needle. The needle was then inserted into the septum of the headspace vials. The needle was then removed while leaving the FEP tubing in the septum as shown in Figure 10. These headspace vials were used as the collection vials. The FEP tubing was then connected to the outlet of the microdialysis probe by a connector. Once the set up was complete the pumps were set at 2.0 μL/ minute. Each sample was performed in triplicate conditions and ran for 30.0 minutes. The samples were then immediately run on the HS-GC-MS (Varian 450-GC). The column used was a Varian Factor Four VF-5ms capillary column with 5% phenylmethylpolysiloxane (length 30 m, 0.25 mm ID, and film thickness of 0.25 μm). The detector utilized was a triple-quad mass spectrometer (Varian 320-MS). The injector temperature was set to 240 °C, the column was set to 35 °C, and the detector temperature was set to 240 °C. The HS-GC-MS was set to scan for a total ion chromatogram from 50-300 *m/z* with a complete run time of 3 minutes.

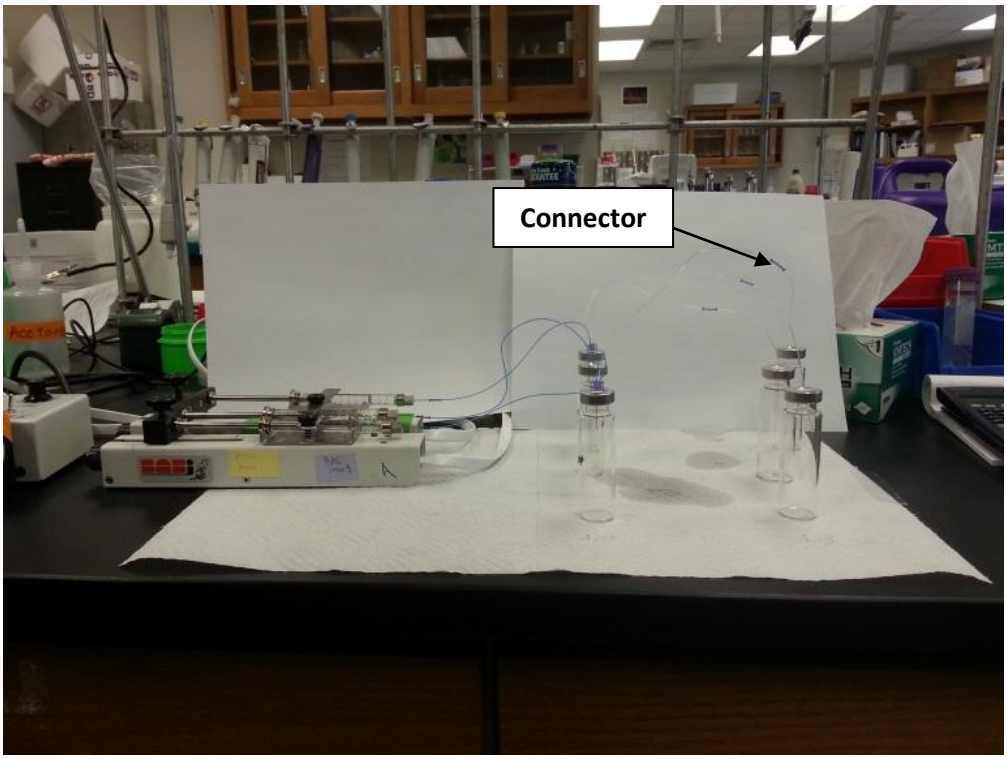


Fig. 8 An example of an in vitro microdialysis setup.

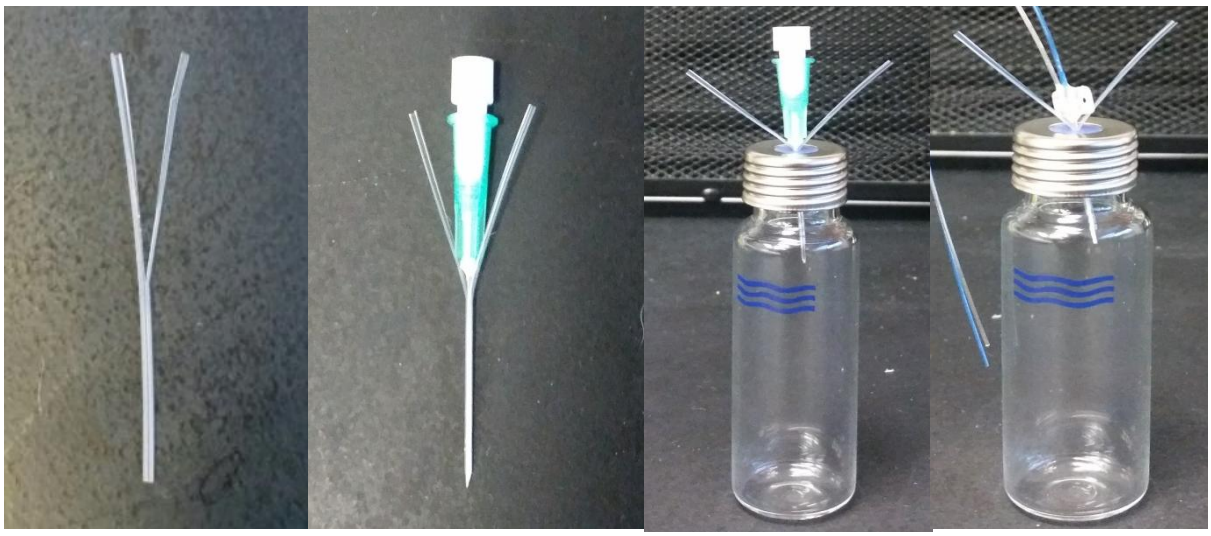


Fig. 9 Pictured above from left to right, an introducer, a needle placed into center of introducer, insertion of introducer, and insertion of the microdialysis probe into the headspace vials.

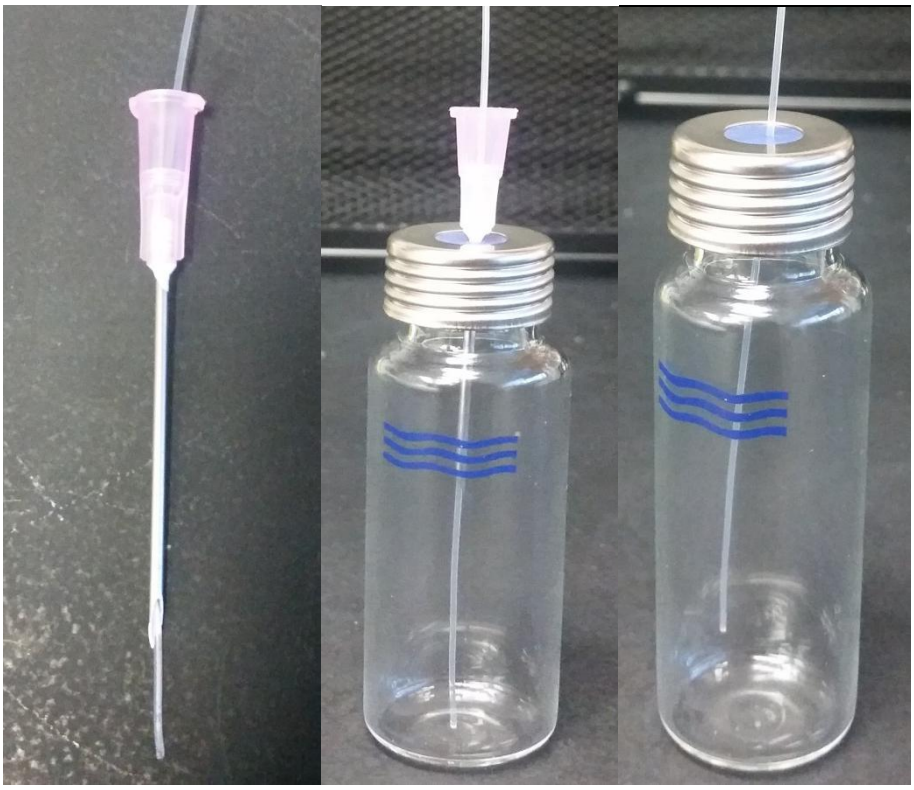


Fig. 10 From left to right. The FEP tubing threaded through the backside of the needle, needle inserted into headspace vial, and needle removed only leaving FEP tubing in the septum.

Isoflurane and DDFP Absorption into Cap

Three 20.0 mL glass headspace vials were capped. These capped headspace vials were then taken into the cold room and injected with 10.0 µL of isoflurane. The cold room (2.8 ˚C) was used to prevent any loss of isoflurane during the transition of isoflurane to the headspace vial. The headspace vials were then taken back to sit at room temperature (20- 22 ˚C) for 30 minutes to completely volatilize. After the compound was had visibly evaporated the first injection of isoflurane was made into the injector port. Gas chromatography (GC-2014, Shimadzu Corporation) with a flame ionization detector (FID) was used. All the injections of isoflurane into the injector port of the GC were 1.0 µL manual injections. The injector temperature was set at 280°C, the column at 60°C, and the detector at 280°C. The column used was a 5% phenyl polysilphenylene-siloxane column (SGE column, 30.0 m X 0.25 mm ID-BPX5 X 0.25 μm). Samples were analyzed every hour from hour zero (initial creation of sample) to six. This same procedure was performed for DDFP.

Separation of Isoflurane and DDFP

Nine 20.0 mL screw cap glass headspace vials were capped. Three glass headspace vials were injected with 10.0 µL of DDFP with a 10.0 µL glass gas-tight syringe. Three more glass headspace vials were injected with 10.0 µL of isoflurane with a 10.0 µL glass gas-tight syringe. The last three glass headspace vials were injected with 10.0 µL of DDFP and 10.0 µL of isoflurane with a 10.0 µL glass gas-tight syringe. The injections were all completed in the cold room (2.8˚C) to prevent any loss during the transition of DDFP and isoflurane to the glass headspace vials.

For the separation of DDFP and isoflurane the same GC-FID and the same parameters mentioned earlier were used. The injection volume for the two pure compounds and for the mixture of the compounds was 1.0 µL with a 10.0 µL glass gas-tight syringe.

Collection of Isoflurane and DDFP with Oil or Air as a Perfusion Fluid

A microdialysis setup was created as shown in Figure 11. The probe was flushed with safflower oil at 1.0 µL/min for 1 hour. Three 20.0 mL glass headspace vials were capped and 25.0 µL of isoflurane or 100.0 µL of DDFP was injected into the vials with a 100.0 µL gas tight glass syringe in the cold room (2.8˚C). These samples were allowed to sit at room temperature (20-22˚C) to completely volatilize for 30.0 minutes. These vials were labeled "sample vials" 1, 2, and 3. Nine 20.0 mL glass headspace vials were capped and labeled in three sets as "collection vials" hr 1, hr 2 and hr 3. An introducer was placed into one of the collection vials labeled hr 1. Another introducer was also placed into sample vial 1 and immediately the microdialysis probe was inserted into the vial. The outlet tubing was also quickly inserted into the collection vial labeled hr 1 with an introducer. Collection vial hr 1 was changed after the first hour and replaced with collection vial hr 2 for the second hour. Finally the collection vial hr 2 was replaced with collection vial hr 3 for the third hour. The microdialysis probe was then removed and placed into sample vial 2 and the collection vials followed the same procedure for the collection vials for sample vial 1.This experiment was repeated with flow rates of 0.5 µL/min and 3.0 µL/min. All collection vials were then analyzed by the GC-FID method. From each sample 1.0 µL of sample from the headspace was removed and injected into the injector port of the GC. A retention time and a peak area were obtained from the GC chromatogram. . A calibration curve was constructed according to peak areas obtained. This experiment was also conducted with DDFP instead of isoflurane. This same procedure was carried out for air as a

perfusion fluid except for only the first 40 minutes of sampling was taken for analysis and different flow rates were ran. The flow rates chosen to use for air was 10.0 µL/min and 20.0 µL/min. These flow rates were chosen based upon a previous experiment. This experiment consisted of placing the outlet of the microdialysis probe in a 10.0 mL beaker filled with water. Once the outlet of the microdialysis probe was submerged in the water different flow rates were tested to see if air bubbles were produced. The visible appearance of air bubbles allowed for confirmation that we were able to perfuse air through the microdialysis probe. Any flow rates lower than 10.0 µL/min was unable to produced sufficient air bubble production.

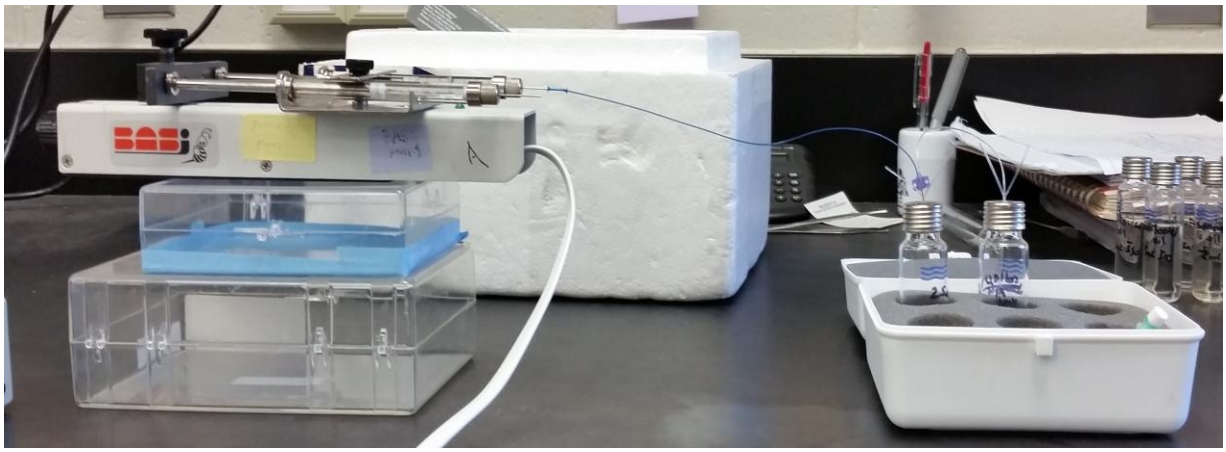


Fig. 11 A microdialysis setup for the different perfusion fluids, oil or air.

Partition Coefficient of Isoflurane and DDFP

Six 20.0 mLglass headspace vials were filled with 5.0 mL of safflower oil. These glass headspace vials were then capped. Once capped the vials were taken into the cold room and injected with two different concentrations (10.0 µL and 20.0 µL) of isoflurane by inserting the needle of a glass gas tight syringe into the septum of the vials. These samples were then immediately vortexed and left to sit at room temperature for two hours. Equilibrium was determined once the peak areas remained constant at hour 5. Once equilibrium was established, 1.0 µL of the headspace from these vials was extracted by using a 10.0 µL glass gas tight syringe. This 1.0 µL headspace sample was then injected into the GC-FID. A calibration curve was created according to the peak areas obtained from the samples. This same procedure was also conducted for DDFP. The partition coefficient was calculated from the average peak areas from the triplicate samples in the first hour.

Results and Discussion

Detection of DDFP

In Figure 12 the GC-MS chromatogram shows DDFP with a retention time of 1.47 minutes with an end run time of 3.0 minutes. The mass spectrum obtained in Figure 13 displays the common fragments of DDFP. The common fragments for DDFP are 69 kDa and 118 kDa. The 69 kDa fragment represents CF_3^-. The 118 kDa fragment represents $C_2F_5^-$. A cross reference was done with the NIST/EPA/NIH mass spectral library (2005 version) to verify that indeed this was DDFP. The graph shown in Figure 14 demonstrates the linear relationship between the changes in concentration within the vial resulting in a specific peak area vs. the concentration external to the probe. With the use of GC-MS DDFP has been identified from the collection samples when using microdialysis sampling.

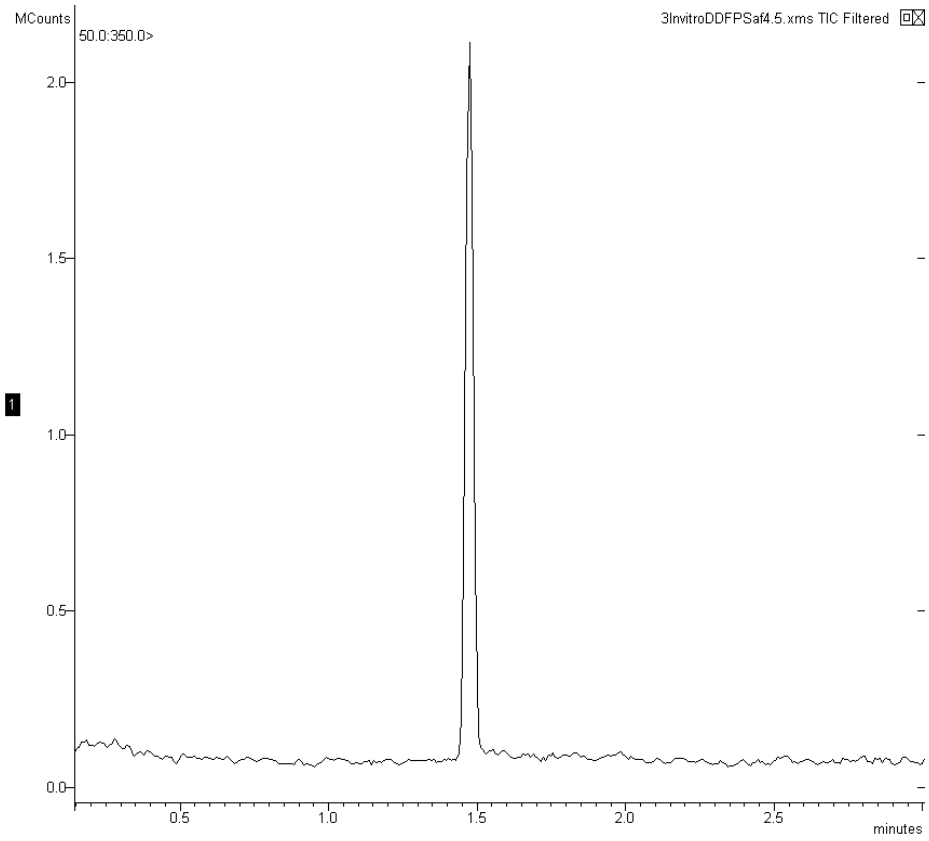


Fig. 12 GC-MS chromatogram of DDFP having a retention time of 1.47.

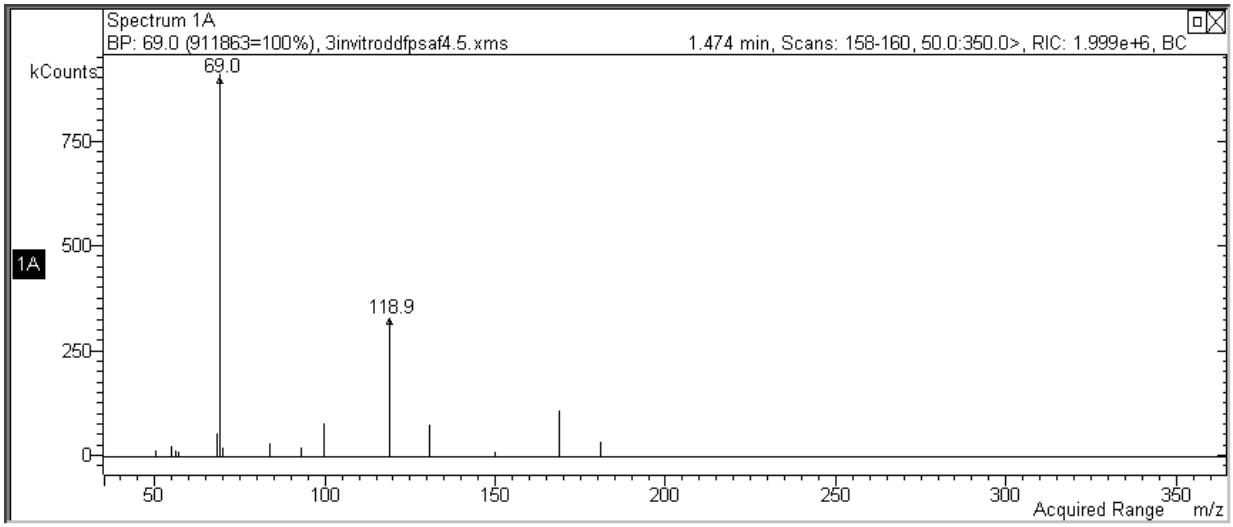


Fig. 13 This is an electron impact (EI) MS of DDFP with a retention time of 1.47 minutes. This was determined to be DDFP because of the two fragments 69.0 and 118.9 *m/z* which are the two most common fragments for DDFP.

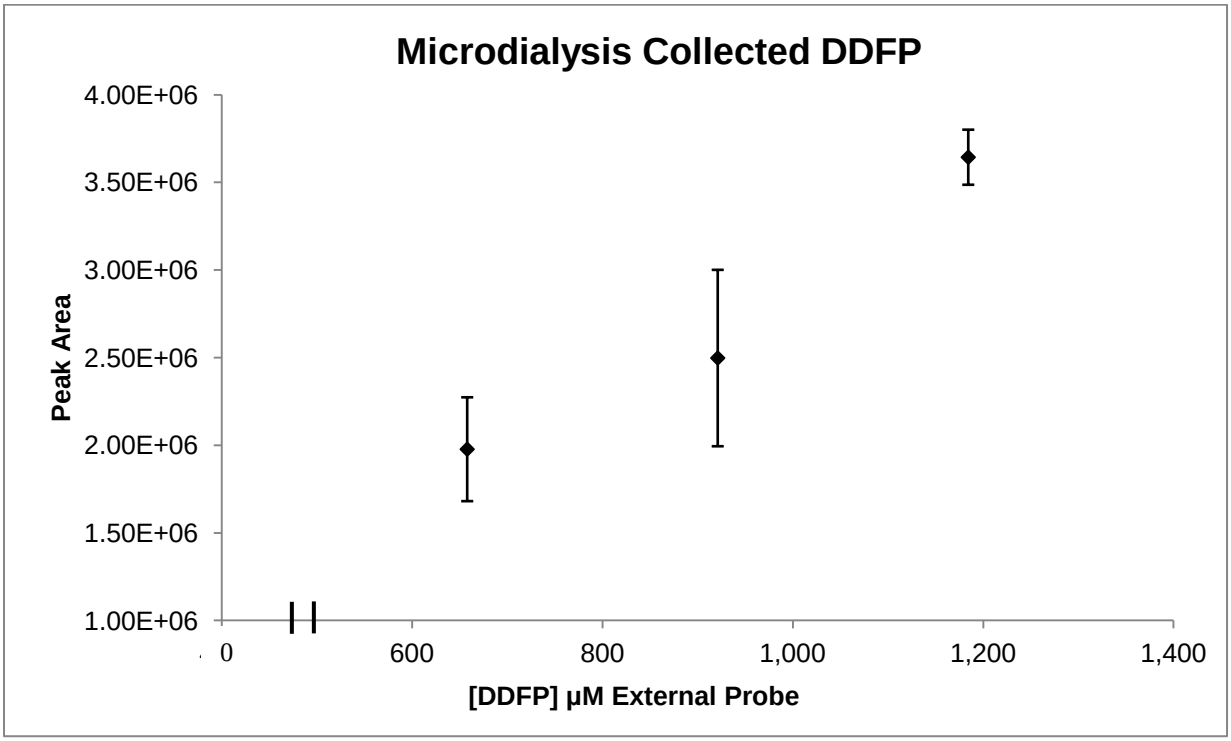


Fig. 14 Three different concentrations of DDFP were analyzed by GC-MS to determine whether or not collection of DDFP was possible with oil as a perfusion fluid. The peak areas increased with the larger concentrations that were external to the probe. N=3

Isoflurane and DDFP Absorption into Cap

From this point on all experiments were performed using a GC-FID instead of the GC-MS mentioned previously. The experiment with the GC-MS was not reproducible even with same sample injections. This was believed to be due to the automatic injection lacking a gas tight syringe. When working with VOC's it is imperative to work with gas tight syringes to prevent loss of sample. VOC's have been known to absorb into plastics.[31] To clarify whether or not isoflurane or DDFP were leaking out of the headspace vial or absorbing/adsorbing into the cap or glass of the headspace vials a time experiment was conducted. This experiment was performed over a time course of six hours. At each hour the same vial was sampled. Figure 15 shows a plot of the output (peak area) vs. time for both solutes. There was no difference in the peak area with respect to time as determined by a repeated measures ANOVA (analysis of variance) with a 95% confidence interval.

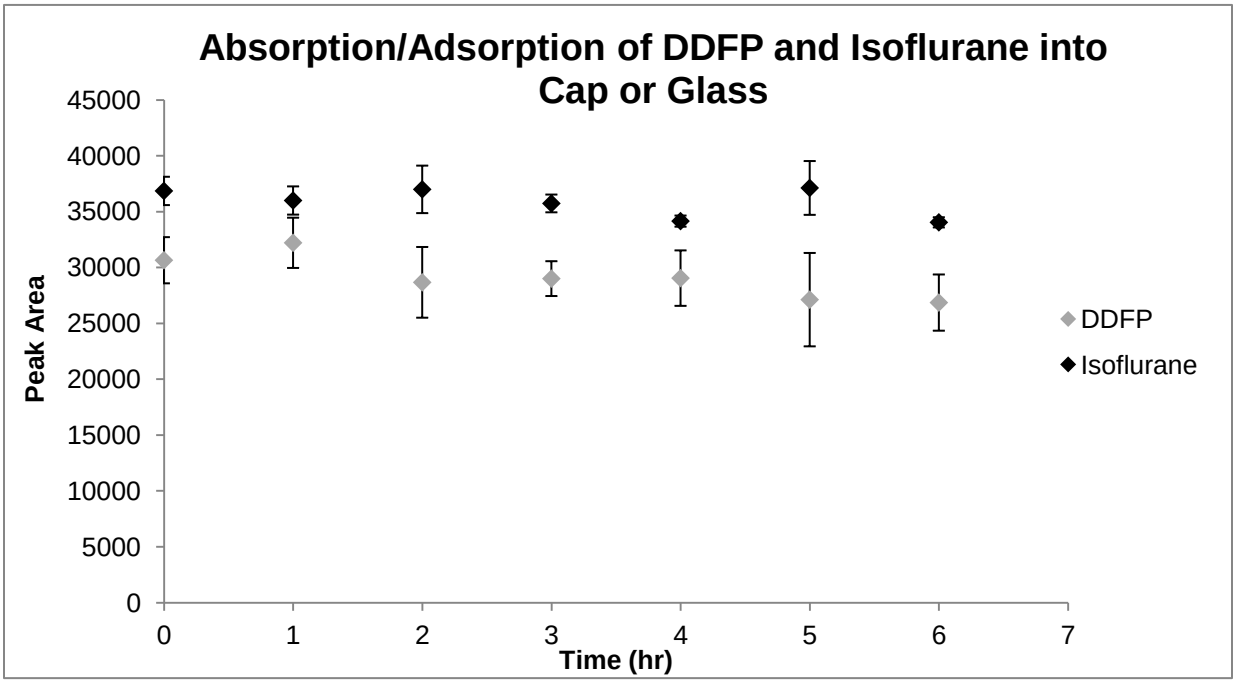
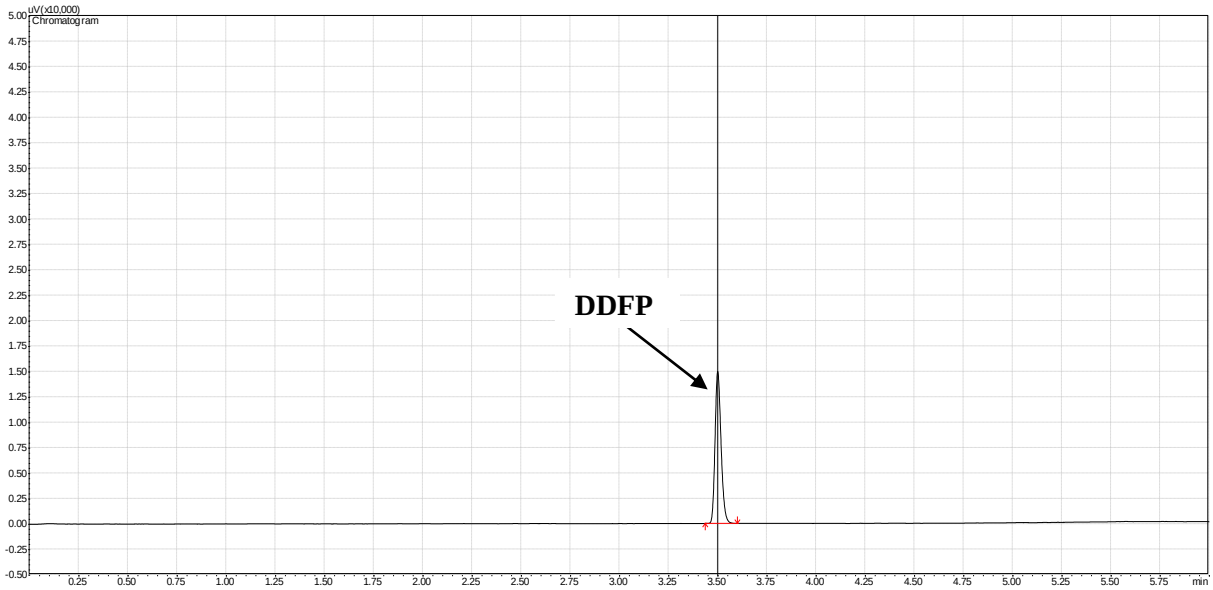


Fig. 15 represents peak area vs. time. The peak areas were obtained from a GC-FID. Peak area displayed no significant change with respect to time determined by a repeated measures ANOVA at a 95% confidence interval between hours. N=3

Separation of Isoflurane and DDFP

The ability to clearly separate isoflurane from DDFP was accomplished by a GC-FID. The injector temperature was set at 280°C, the column at 60°C, and the detector at 280°C using the software GC solutions. In Figure 16A, the chromatogram shows that DDFP has a retention time of 3.5 and in Figure 16B isoflurane has a retention time of 3.7. In Figure 17 the two compounds are shown to be separated. The calculated resolution for the two compounds was 1.2. Since isoflurane is the most common anesthetic for animals it will be expected that isoflurane will be collected and detected simultaneously in vivo with DDFP. The ability to separate these two compounds with good resolution allows for the proper determination of concentration of these two compounds and the continuation of *in vivo* work

A



B

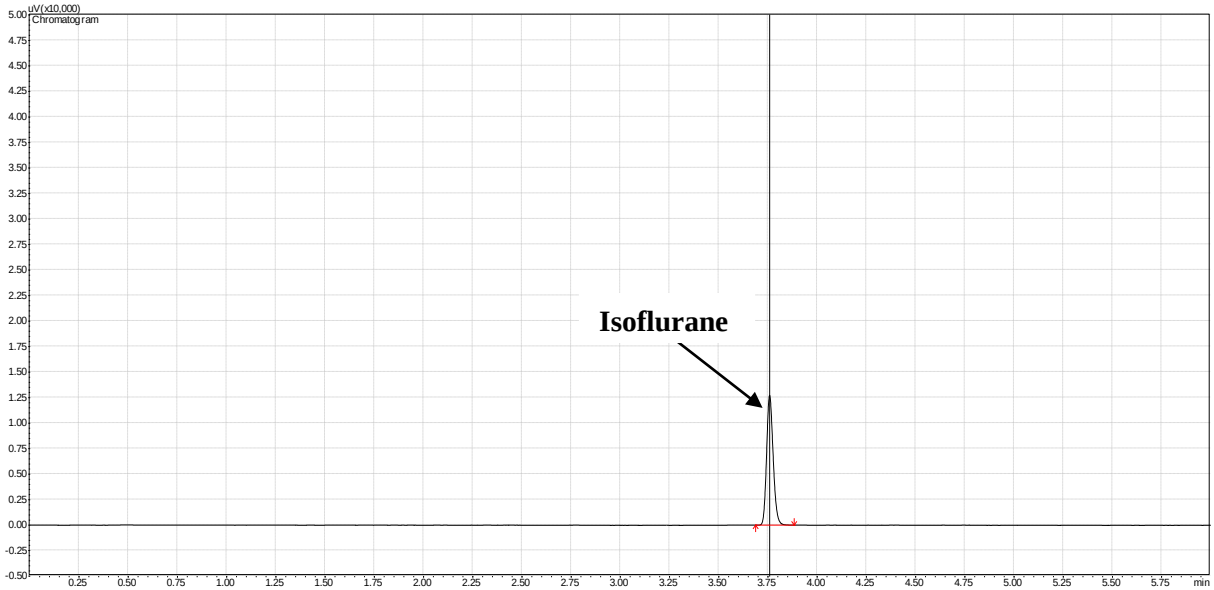


Fig. 16 A GC chromatogram of DDFP and isoflurane A) neat DDFP with a retention time of 3.5 B) neat isoflurane with a retention time of 3.7.

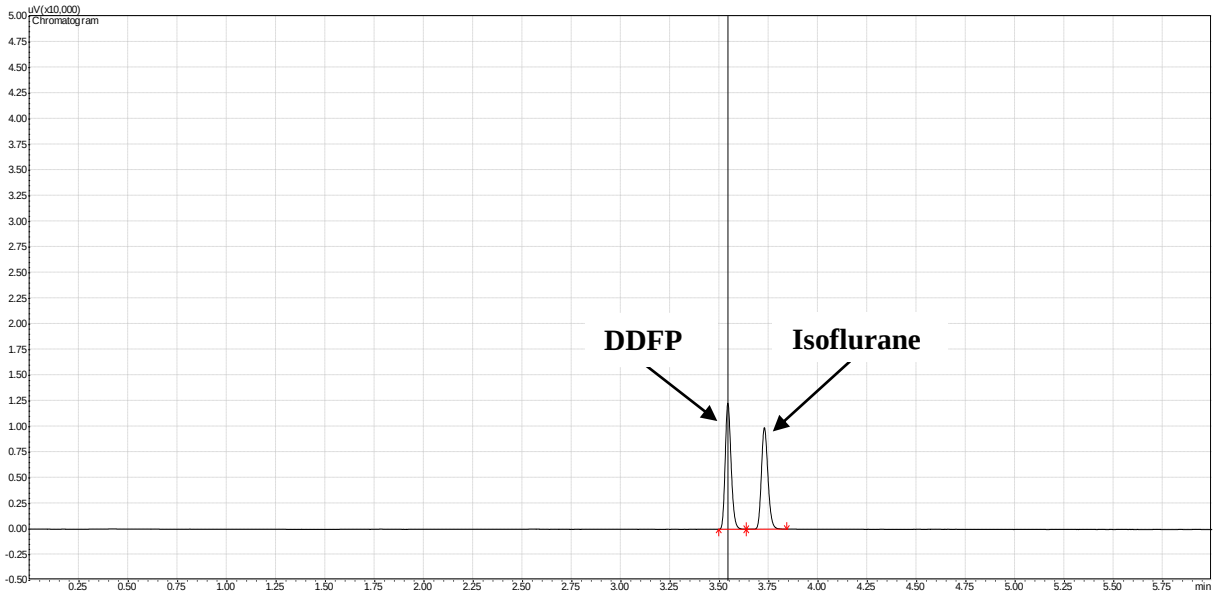


Fig. 17 A GC chromatogram of a mixture of isoflurane and DDFP.

The Flux of Isoflurane and DDFP with Oil as a Perfusion Fluid

When using oil as a perfusion fluid, the flux obtained for isoflurane at 0.5 µL/min for hour 1 was165±10 nmol/min, hour 2 was 124±40 nmol/min, and hour 3 was 73±40 nmol/min. When the flow rate was switched to 1µL/min the flux for hour 1 was 38±10 nmol/min, hour 2 was 26±3 nmol/min, and hour 3 was 16±6 nmol/min. With a flow rate of 3.0 µL/min the flux was 50±10 nmol/min at hour 1, 35±10 nmol/min at hour 2 and 27±10 nmol/min at hour 3. (Figure 18) It was determined that there was a significant difference with isoflurane with respect to time and flow rates by a two factor ANOVA at a 95% confidence interval. The significance was found between the flow rates, 3.0 µL/min and 1.0 µL/min, at hour 1 and hour 2. There was significance between the flow rates, 3.0 µL/min 0.5 µL/min, at hour 1 and hour 2. This significance of flux between flow rates can be identified with a lower flow rate a higher concentration of isoflurane can be obtained. The fact that at 3.0 µL/min is higher than 1.0 µL/min can be explained because at higher flow rates a higher mass recovery is obtained. There is also significance between hour 1 and hour 3 for flow rate of 0.5 µL/min. This is most likely due to the gradual depletion of the sample from the headspace vial.

The flux obtained for DDFP with a flowrate of 0.5 µL/min was 16±7 nmol/min for hour 1, 16±2 nmol/min for hour 2, and 16±7 nmol/min for hour 3. With a flowrate of 1.0µL/min the flux was 22±2 nmol/min, hour 2 was 26±20 nmol/min, and hour 3 was 13±9 nmol/min. With a flowrate of 3.0 µL/min the flux was 14±2 nmol/min for hour 1, 21±9 nmol/min for hour 2, and 13±3 nmol/min for hour 3. (Figure 19) There was no significant difference with DDFP with respect to time and flowrate by a two factor ANOVA at a 95% confidence interval. These low fluxes could be due to the fluorinated ethylene propylene (FEP) tubing. Organic molecules are notorious for absorbing into plastics.[31] The perfusion fluid may not have been the correct option

for DDFP. Another option is that this compound could be absorbing/adsorbing to the membrane of the microdialysis probe.

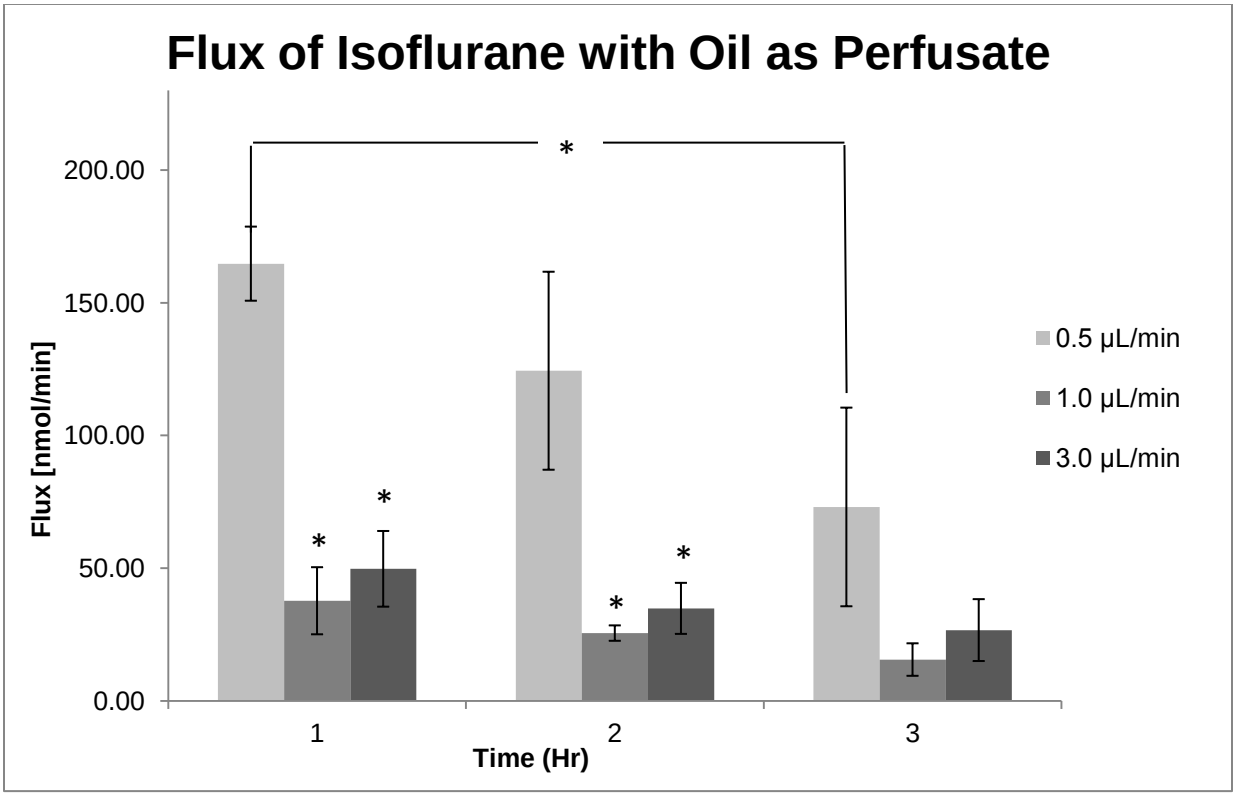


Fig. 18 The flux of Isoflurane at different flow rates and time. There was a significant difference between hour 1 and 3 for the 0.5 µL/min flow rate. At flow rates of 1.0 µL/min and 3.0 µL/min the flux was significantly different from 0.5 µL/min at hours 1 and 2. The significance was determined by a two factor ANOVA at a 95% confidence interval. N=3

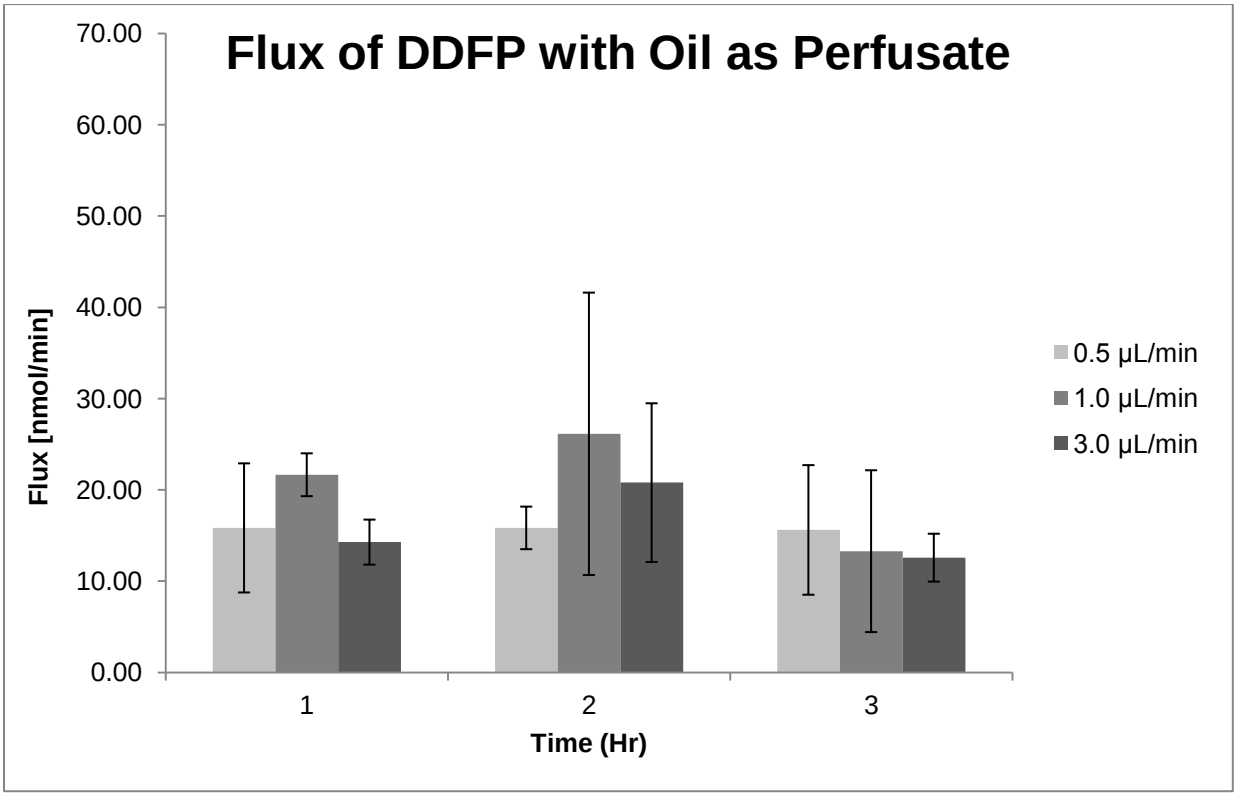


Fig. 19 The flux of DDFP at different flow rates vs time. There was no significant difference with flux with respect to time and flowrate for DDFP as determined by a two factor ANOVA at a 95% confidence interval. N=3

The Flux of Isoflurane and DDFP with Air as a Perfusion Fluid

When substituting air as a perfusion fluid the flux at 10 μL/min was163±50 nmol/min and at 20 μL/min 353±80 nmol/min for isoflurane. (Figure 20) There was a significant difference between flow rate 10 μL/min and 20 μL/min as determined by a t-test ($p<0.025$) at a 95% confidence interval.

The flux for DDFP at 10 μL/min was 99±9 nmol/min and at 20 μL/min was 148±4 nmol/min. (Figure 19) There was a significant difference between the flow rates of 10 μL/min and 20 μL/min as determined by a t-test ($p=0.001$) at a 95% confidence interval. The higher the flow rate the higher the flux obtained for both compounds. This might be a factor of obtaining higher mass recovery at higher flow rates.

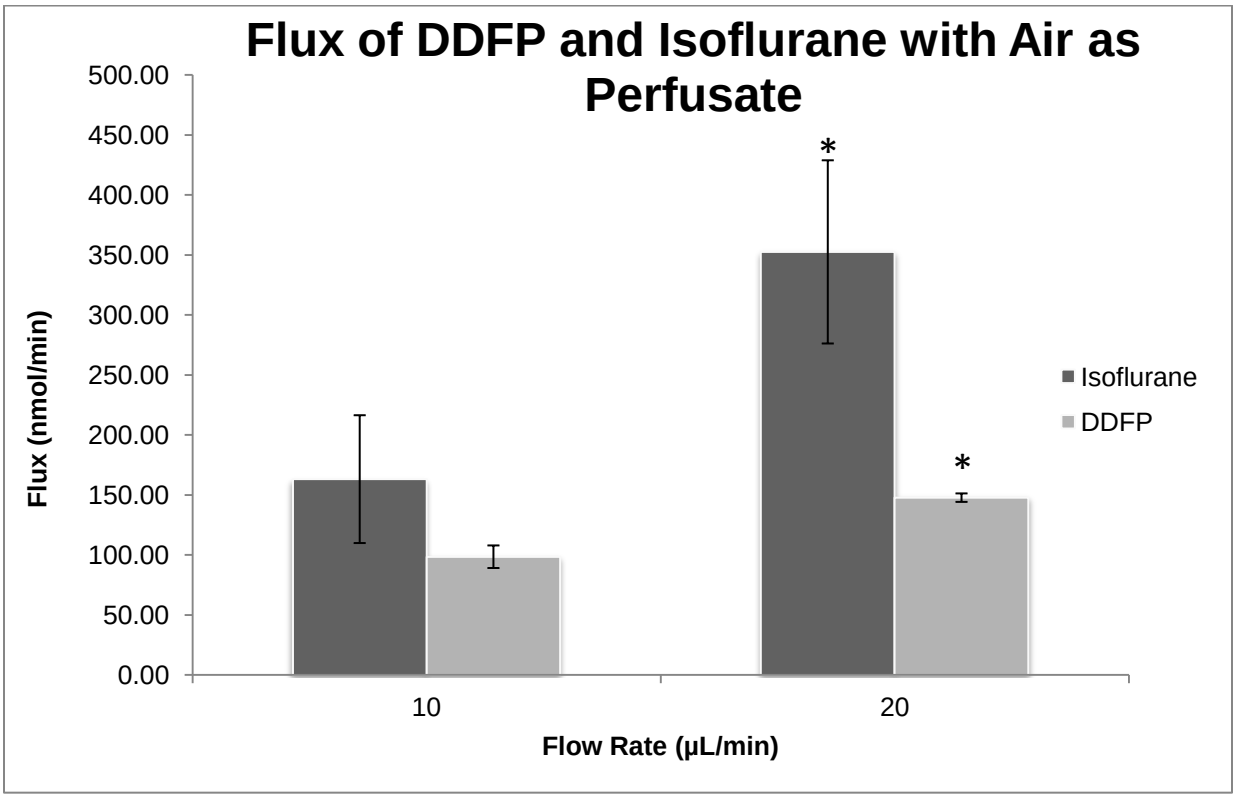


Fig. 20 The flux of isoflurane and DDFP with air as a perfusion fluid at different flow rates. There was a significant difference in flux with increased flow rates for both compounds as determined by t-test at a 95% confidence interval. N=3

Oil/Air Partition Coefficient of Isoflurane and DDFP

Equilibrium of DDFP and isoflurane were determined before the partition coefficient was calculated by observing shift in peak area at Hr 1 and Hr 5. In Figure 21 and Figure 22 the peak areas remain consistent for DDFP and isoflurane. This consistency denotes that the compounds have reached equilibrium. The $K_{Oil/Air}$ of isoflurane at 25˚C was calculated to be 10.62 from the average peak areas from the two concentrations (16.22 mM and 32.43 mM) as shown in Table 1. This partition coefficient was reported in the literature value as 98. [32] This can most likely be explained by not using the same temperature as in the literature (37˚C) and also not using olive oil. This value still agrees that isoflurane partitions more into oil vs. air. The oil air partition coefficient of DDFP at 25˚C was determined to be 0.53 from the average peak areas from the two concentrations (11.31 mM and 22.64 mM) as shown in Table 2. There is currently no oil/air partition coefficient for DDFP in the literature. This K value denotes that DDFP partitions more into air vs. oil.

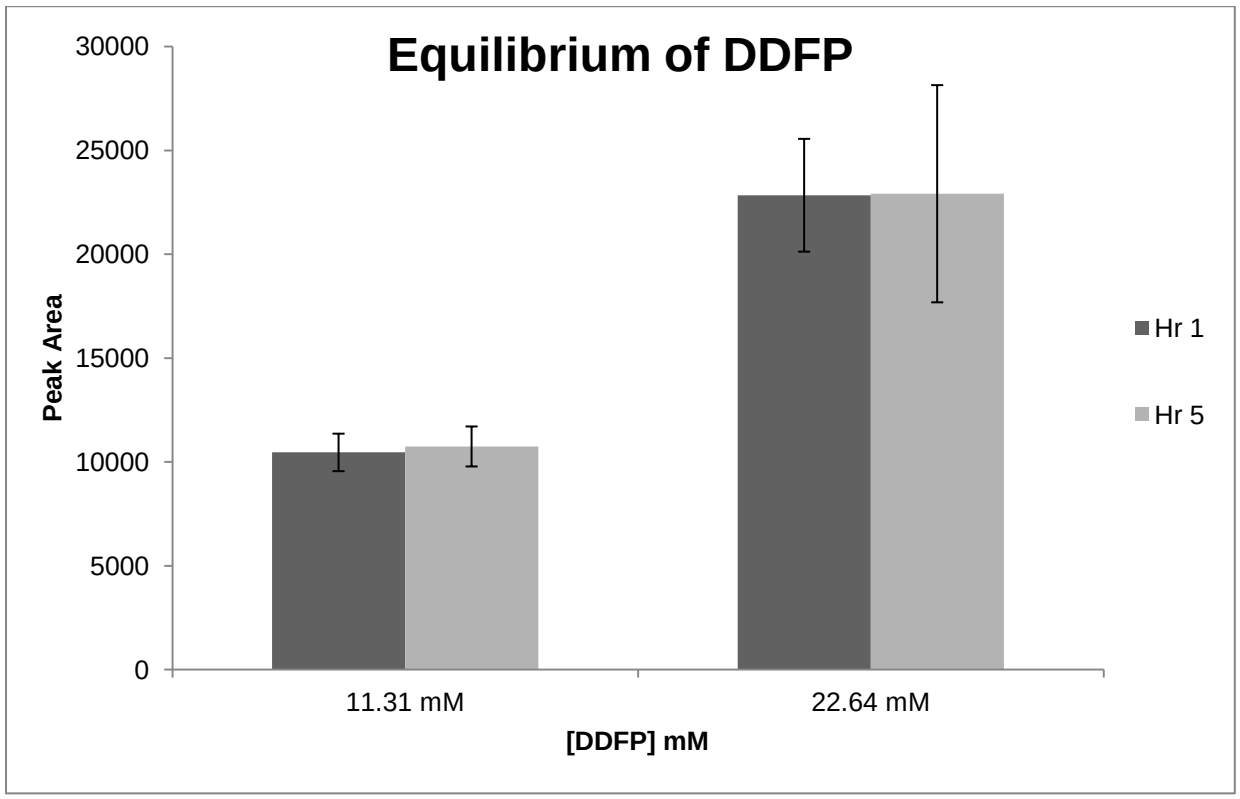


Fig. 21 DDFP's peak area remains consistent from hour 1 to hour 5 for two different concentrations. N=3

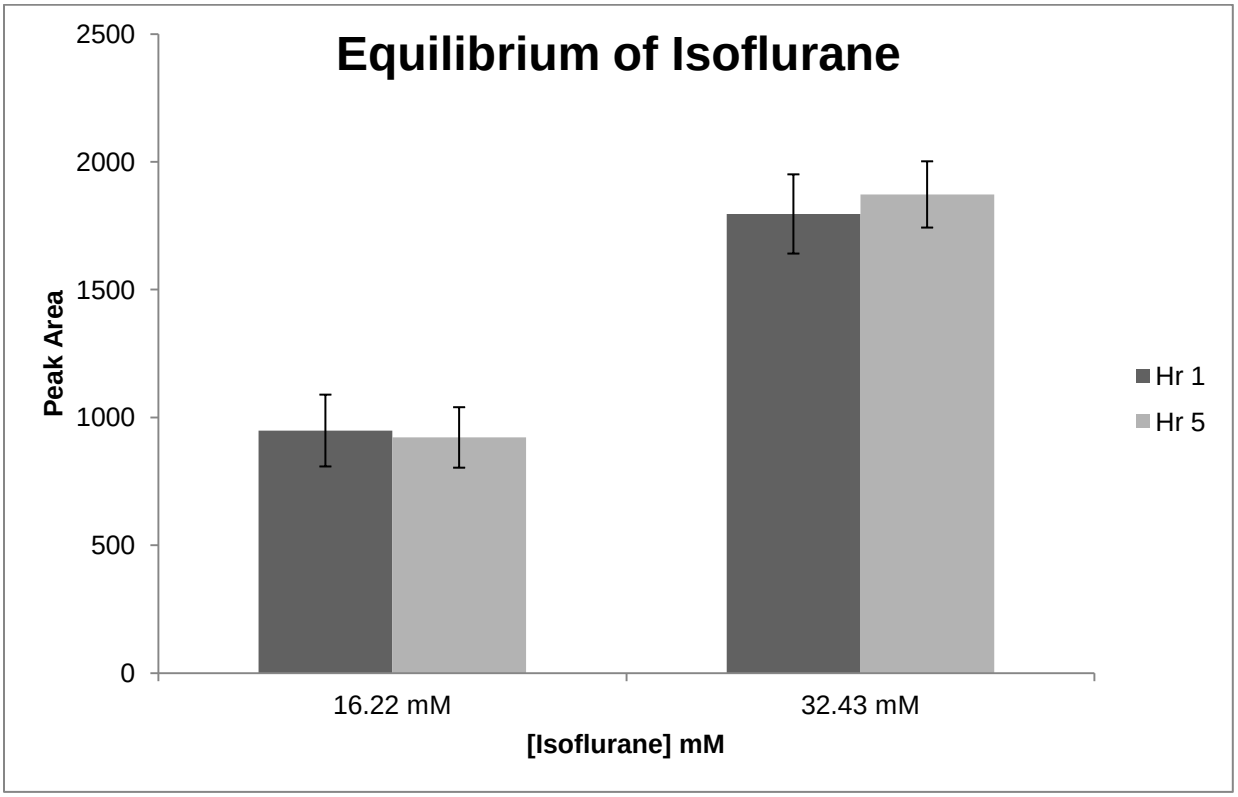


Fig. 22 Isoflurane's peak area remains consistent from hour 1 to hour 5 for two different concentrations. N=3

Table 1
Oil/Air Partition Coefficient of DDFP

Temperature	Concentration (mM)	Phase Ratio (β)	Average Peak Area	Partition Coefficient (K)
25°C	11.31	3	10747±903	0.73
25°C	22.64	3	22840±2715	0.22

Table 2
Oil/Air Partition Coefficient of Isoflurane

Temperature	Concentration (mM)	Phase Ratio(β)	Average Peak Area	Partition Coefficient (K)
25°C	16.22	3	922±140	10.87
25°C	32.43	3	1796±155	10.78

Conclusion

VOC's can be collected via microdialysis sampling technique *in vitro*. The fluxes of these compounds were calculated from different flow rates. The microdialysis setup needs to be optimized due to possible loss of analyte. For instance the use of FEP tubing could be a cause of loss of analyte. By replacing this tubing with a fused silica tubing could increase sample recovery. Also finding a viable perfusion fluid to collect these VOC's is important. Oil seemed to be an option for isoflurane but not for DDFP. Air was a better choice for DDFP hinting that DDFP would have a lower partition coefficient. There are many parameters that can be changed within the microdialysis setup presented here. Microdialysis can collect these compounds but further investigation needs to be continued. The oil/air partition coefficients for isoflurane and DDFP have been identified at 25˚C. DDFP has currently no reported value for an oil/air partition coefficient.

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Table 3
Equilibrium of DDFP

DDFP	Concentration (mM)	Peak Area Hr 1	Peak Area Hr 5
Sample 1	11.31	11247	11449
Sample 2	11.31	10659	11143
Sample 3	11.31	9475	9649

DDFP	Concentration (mM)	Peak Area Hr 1	Peak Area Hr 5
Sample 1	22.64	20008	18929
Sample 2	22.64	23093	28834
Sample 3	22.64	25420	20978

Table 4
Equilibrium of Isoflurane

Isoflurane	Concentration (mM)	Peak Area Hr 1	Peak Area Hr 5
Sample 1	16.22	1036	832
Sample 2	16.22	787	878
Sample 3	16.22	1024	1056

Isoflurane	Concentration (mM)	Peak Area Hr 1	Peak Area Hr 5
Sample 1	32.43	1726	1789
Sample 2	32.43	1689	1807
Sample 3	32.43	1974	2022