TOWARDS FORENSIC APPLICATION OF SOLID-PHASE MICROEXTRACTION

Development and validation of an SPME approach to study pharmaceuticals and illicit drugs

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colofon

Towards forensic application of solid-phase microextraction. Development and validation of an SPME approach to study pharmaceuticals and illicit drugs.

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Development and validation of an SPME approach to study pharmaceuticals and illicit drugs

"Solid-phase microextraction" als toekomstige bemonsteringstechniek in forensisch onderzoek (met een samenvatting in het Nederlands)

Proefschrift

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INTRODUCTION

BACKGROUND

Essential in forensic toxicology is the detection and evaluation of postmortem drug concentrations to determine whether a compound can be identified as the cause of death or a contributor to the cause of death. However, postmortem drug concentrations do not necessarily reflect the concentration at the time of death and vary according to the sampling site and time interval between death and sample collection. A major problem in chemical analysis in forensics studies is that the amount of biological sample is often limited. Moreover, it is not possible to perform repeated sampling after death without disturbance of the biological system. Roughly half of the 57 drugs of abuse that are screened routinely at the Netherlands Forensic Institute are predominantly ionized at physiological pH. Whereas the partition behavior and sampling methodology for neutral toxicants is reasonably well documented, understanding the distribution processes of ionizable compounds and interpreting sampling data poses various scientific challenges.

The major aim of this thesis is to develop new analytical techniques that can be applied in postmortem redistribution studies of drugs and pharmaceuticals. To accomplish this, a sampling method using solid-phase microextraction (SPME) was investigated and optimized for a selected number of compounds, specifically focusing on ionizable drugs of abuse.

In this chapter, forensic toxicology is introduced. Special attention is given to the uptake, distribution, metabolism and elimination of compounds, and in particular ionizable compounds. These processes are also discussed in the light of a postmortem situation, which leads to the introduction of the phenomenon of postmortem redistribution. Current analytical tools in forensic studies are highlighted, with special attention for solid-phase microextraction.

FORENSIC TOXICOLOGY

Forensic toxicology is a discipline in toxicology that aids in the legal investigation of death, poisoning or drug use. Most important in this field is the collection of data and the interpretation of the results. At the Netherlands Forensic Institute, most toxicological cases are concerned with driving under the influence of either alcohol or drugs. In 2014, more than 3000 cases were reviewed by the forensic toxicology department (figure 1). While only a small

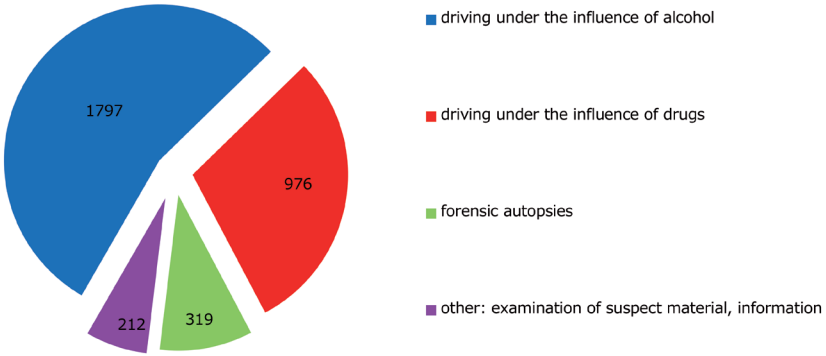

Figure 1. Overview of forensic toxicology cases in the Netherlands, as recorded by the Netherlands Forensic Institute. Report year: 2014

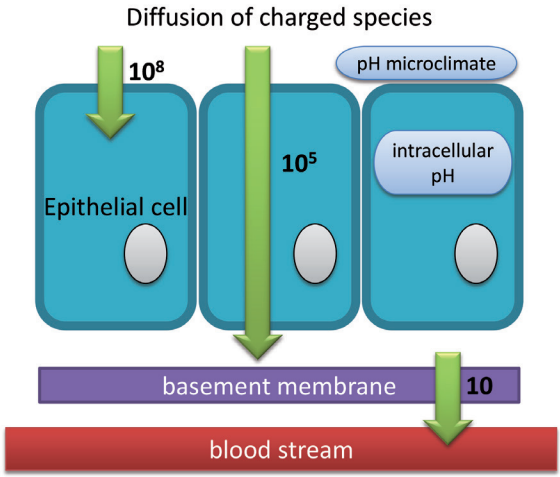

Figure 2. Diffusion of charged chemicals is possible across phospholipid membranes (left arrow), epithelium (middle arrow), and basement membrane (right arrow). The numbers indicate the fold increase in permeability of the neutral versus the charged species of an ionizable compound (*Avdeef, 2012*). Also, the uptake of charged chemicals can be altered by the presence of a pH microclimate and differences in intracellular pH.

portion of the total number of cases concerned forensic autopsies and their outcome, the complexity of postmortem toxicology makes it a large part of the work of a forensic toxicologist.

Postmortem toxicology

The interpretation of toxicological results is mostly based on pharmacokinetic and pharmacodynamic data. In clinical toxicology, or antemortem, this data on concentration-effect relations is readily available. For pharmaceuticals, the deposition of the compound within the body is well studied. Deposition is defined by a number of processes, including absorption, distribution, metabolism and elimination (ADME in short). In a postmortem situation, changes can occur in these processes. The following paragraphs describe the ADME processes, with potential postmortem changes.

Absorption

Absorption describes the uptake of the compound into the bloodstream and, together with metabolism, determines the bioavailability. The route of administration is an important consideration in absorption. Most pharmaceuticals are administered orally, and need to be taken up from the gastrointestinal tract. The uptake is mainly driven by passive diffusion, and requires ionizable compounds to be in their neutral form. This is also known as the pH partitioning hypothesis (*Avdeef 2012*). The uptake of ionizable compounds is therefore dependent on the pH inside the gastrointestinal tract. Due to the large pH gradient in the gastrointestinal tract the uptake of ionizable chemicals is highly location-specific. Uptake of anionic compounds mostly occurs in the stomach, whereas cationic compounds are taken up in the colon (*Avdeef 2012*).

However, there are some exceptions to the pH partitioning hypothesis, which allow for the diffusion of the charged form of a compound (figure 2).

First of all, passive diffusion is not solely reserved to the neutral form of a chemical. The charged species of a compound is capable of passive diffusion, albeit at much lower rates than for the neutral species. Figure 2 shows the fold increase in permeability of the neutral form of a chemical over the charged form over different intestinal barriers (*Avdeef 2012*). The permeability of the neutral form of a chemical is 10^8 greater than for the charged form for the transport across a phospholipid bilayer. For the epithelium itself, this factor is 10^5, in part due to paracellular transport and ion-selectivity in the tight

junctions. For the basement membrane, ion-selectivity reduces the difference between neutral and ionized permeability to a factor 10. Secondly, the pH close to the epithelium might be different than the pH in the lumen itself. The microclimate pH in the intestine can be up to two log units lower than the luminal pH, most likely created by the amphoteric nature of the mucus layer (*Shiau et al. 1985*). In the stomach, reported microclimate pH is 8.0, whereas the normal pH of the stomach is 1.7 in fasted condition (*Rechkemmer 1991*). Here, the secretion of HCO_3^- from the gastric epithelium greatly influences the microclimate pH (*Rechkemmer 1991*). Lastly, intracellular pH also affects absorption. Within a cell, pH can be as low as 4.5 in lysosomes and as high as 8.0 in mitochondria (*Asokan & Cho 2002*). This can have a dramatic effect on charge and physicochemical properties of weak acids and bases. Specifically, ionizable compounds can enter the cell as a neutral compound, but become charged inside the cell due to the difference in intracellular pH. In its charged form, the compound cannot diffuse as effectively out of the cell, leading to accumulation inside the cell. This is known as ion trapping.

In forensic toxicology, one should be aware that illicit drugs usually have a different or unconventional route of administration compared to pharmaceutical drugs. Examples of these types of administration routes include smoking, snorting and self-injecting drugs. The route of administration can affect uptake, blood concentration and rate of metabolism, thereby affecting total body disposition (*Drummer 2007*). Postmortem, passive diffusion continues, and so does absorption. An example of this is after an overdose of an orally ingested drug. Death might occur before absorption is complete, leading to higher concentrations in blood and tissue near the stomach than elsewhere in the body, due to ongoing absorption (*Ferner 2008*). Other factors contributing to changed absorption are intracellular pH changes and membrane damage. These factors will be discussed later in this chapter.

Distribution

After absorption, the compound reaches the blood stream where it is distributed throughout the body. In the blood, a compound can be freely dissolved in plasma, bound to plasma proteins or bound to membranes (mainly red blood cells).

In plasma protein binding, the most abundant carrier protein in blood is human serum albumin (HSA). This macromolecule binds many different endogenous compounds, including fatty acids, thyroxine and bilirubin (*Ghuman et al.*

2005). Most drugs have a high binding affinity to HSA, specifically to two drug binding sites, Sudlow sites I and II (*Filip et al. 2013*). Site I (also known as the warfarin site) primarily binds dicarboxylic acids and heterocyclic molecules with a negative charge in the center of the molecule (*Filip et al. 2013*). The binding pocket is made up mainly of hydrophobic amino acids with positively charged amino acids around the entrance of the pocket (*Filip et al. 2013*). Site II (the indole-benzodiazepine site) binds carboxylic acids and compounds with the negative charge away from the hydrophobic part of the structure. Binding affinity is strongly dependent on substitutions (with for instance halogens) (*Filip et al. 2013*). As many endogenous and exogenous compounds have affinity for HSA, competition for the binding sites of HSA occurs often (*Filip et al. 2013*). After HSA, α_1-acid glycoprotein (AGP) is the next important drug-binding protein. AGP as carrier protein was first described in 1975 (*Routledge 1986*), but has gained more attention in recent years as certain antiretroviral drugs for the treatment of AIDS show high affinity for AGP (*Israili & Dayton 2001*). AGP is a glycoprotein, consisting of a single polypeptide chain and five glycans (*Filip et al. 2013*). AGP has a high level of heterogeneity due to differences in these glycans (*Filip et al. 2013*). Also, the protein is subject to genetic polymorphisms, which show different binding affinities for some compounds (*Filip et al. 2013*). AGP reportedly has one binding site, which is largely non-polar but contains some positively charged amino acids (*Filip et al. 2013*). Interestingly, binding of basic compounds has been largely attributed to AGP instead of HSA (*Israili & Dayton 2001*). However, not all basic drugs show affinity for AGP, and AGP can also bind acidic and neutral compounds (*Israili & Dayton 2001*). While HSA concentrations in blood do not vary widely, AGP is an acute-phase protein that varies in concentration with age, gender, pregnancy and disease state (*Israili & Dayton 2001*). Postmortem, protein concentrations will decrease as the synthesis of new protein is terminated but proteolysis by autoenzymes (and bacteria in putrefactive cases) continues (*Pélissier-Alicot et al. 2003*).

Binding to membranes does not only influence distribution in blood, but also distribution to solid tissues and organs. Important physicochemical properties that define sorption to membranes include ionization state and hydrophobicity. Hydrophobicity is usually defined using the octanol-water partition coefficient, log P or log K_{ow}. Log K_{ow} is a good predictor for the sorption of neutral compounds to membranes (*Endo et al. 2011*). However, the octanol-water system cannot account for specific interactions between ionized chemicals and biological membranes. The sorption of ionized compounds to phospholipid bilayers is better predicted by the use of membrane-water distribution coefficients (log

D_{mem}). A phospholipid is in essence a zwitterion, with negatively charged phosphates and positively charged trimethylammonium groups (*Avdeef et al. 1998*). Neutral compounds partition into the middle of the membrane, while ionized compounds will sorb to the charged head group of the phospholipid (*Avdeef et al. 1998*). This is termed the pH piston hypothesis, where the location of an ionizable compound inside a membrane is dependent on its charge (*Avdeef et al. 1998*). Antemortem, differences in intracellular pH can affect the distribution of ionizable compounds, as previously discussed. Postmortem pH changes could give rise to changes in the distribution of an ionizable compound in membranes, affecting both blood concentrations and tissue concentrations. This will be discussed later in this chapter.

The distribution of a compound in pharmacology is defined using a single term, the apparent volume of distribution (V_d). This is a theoretical value that indicates the volume necessary to uniformly distribute the total amount of a chemical in the body at the concentration measured in the plasma. A small V_d therefore means that the compound mainly remains in the blood stream, while a large V_d indicates high distribution to tissues (e.g. adipose tissue). However, even drugs with a small V_d can have a high distribution for specific tissues (*Pélissier-Alicot et al. 2003*). In general, V_d will increase with hydrophobicity, and decrease with increased ionization and high plasma protein binding.

A few organs are known for their high concentrating ability antemortem, making these postmortem drug reservoirs. These include (parts of) the gastrointestinal tract, the lungs, the liver and, for some compounds, the myocardium (*Pélissier-Alicot et al. 2003*). Especially lipophilic, cationic compounds are stored in drug reservoirs and have the highest tendency to redistribute postmortem (*Yarema & Becker 2005*). This will be discussed in more detail later in this chapter.

Metabolism

Metabolism of exogenous compounds occurs mostly in the liver, which is why this is an important drug reservoir. Antemortem, metabolic enzymes modify drugs by adding or removing specific groups, usually to make these compounds more polar so they can be excreted via the urine. Phase I metabolic enzymes include the cytochrome P450 system and reactions include oxidation, reduction and hydrolysis. Most of these reactions are active processes or require oxygen for the reaction process. When phase I metabolites are not polar enough for excretion, they require another reaction using phase II enzymes. These are conjugating enzymes, attaching a large inactive group to the compound.

These reactions include glucuronidation, glutathione conjugation, sulfation and acetylation. In forensic cases involving drug overdose, metabolic enzymes might be saturated by high drug concentrations. This may lead to a decreased rate of elimination of the drug from the body, and with that prolonged effects of the compound (*Sue & Shannon 1992*).

Postmortem, metabolic enzymes lose their function, most likely due to a lack of energy sources and/or oxygen. A study on enzyme activity in postmortem rat liver microsomes showed a 90% decrease in P450 enzyme and glutathione S-transferase activity after 48 hours (*Yamazaki & Wakasugi 1994*). However, UDP-glucuronyl tranferase activity only decreased 30% over this time frame. Others reported a continuing activity of some phase I reactions, specifically hydrolysis by esterases as described for the metabolism of cocaine (*Moriya & Hashimoto 1996*). Some metabolic processes might still occur postmortem, so metabolism should still be considered for at least specific compounds, as highlighted here for the metabolism of cocaine.

It is important to distinguish physiologically-based drug metabolism from drug degradation by putrefactive processes. With the onset of putrefaction and a loss of immune functions, bacteria will proliferate and feed on available proteins and chemicals. An example of microbial drug degradation is the metabolism of nitrobenzodiazepines. The presence of 7-amino metabolites indicates microbial bioconversion of these types of compounds (*Skopp 2010*). Besides drug degradation, neo-formation is also the result of microbial processes. An example of neo-formation is the postmortem synthesis of ethanol and other higher alcohols. High postmortem ethanol concentrations may therefore not be caused by ethanol intake antemortem. During interpretation, the presence of higher alcohols indicates postmortem formation of ethanol (*Skopp 2010*).

Elimination

Exogenous compounds, either metabolized or not, are mainly eliminated from the body via the urine or the feces. Urinary excretion occurs via the kidney. Compounds are filtered from the blood by glomerular filtration and tubular secretion. Through tubular reabsorption, compounds can be reabsorbed from the urine to the blood. Postmortem, both glomerular filtration and tubular secretion cease (*Pélissier-Alicot et al. 2003*). Glomerular filtration is dependent on the afferent blood flow, while tubular secretion is an active process. Tubular reabsorption is based on passive diffusion, and can continue postmortem for several hours (*Pélissier-Alicot et al. 2003*).

Glucuronide and other drug conjugates and compounds with high molecular weight are mainly excreted via the feces. These compounds are concentrated in the bile via active processes. Postmortem, these will no longer occur. Also the storage of bile and emptying of the gall bladder are active processes and will consequently cease postmortem (*Pélissier-Alicot et al. 2003*).

Postmortem redistribution of drugs

Postmortem redistribution is in principle the postmortem diffusion of drugs along a concentration gradient, from sites of high concentration in organs to the blood, leading to an artefactual increase in blood concentrations. This leads to major challenges in the interpretation of postmortem obtained concentrations in blood and tissue, and has therefore been called "a toxicological nightmare" (*Pounder & Jones 1990*). There are many factors that contribute or influence postmortem redistribution, these will be discussed below.

Postmortem physiological changes

Initiation of postmortem physiological changes already occurs during the agonal phase, just before death. A decrease in cardiac output and blood supply, combined with hypotension, impaired ventilation and dehydration eventually lead to hypoxia (*Skopp 2010*). Postmortem, without proper respiration, the production of ATP ceases and anaerobic metabolism starts. This leads to a build-up of lactic acid thereby decreasing the intracellular pH (*Yarema & Becker 2005*). Body pH decreases to pH 6.1 within 24 hours of death, with a further decline to pH 5.1 after 96 hours (*Donaldson & Lamont 2013*). Lactic acid levels can increase up to 10 mmol/L, 20 to 40 times higher than antemortem levels (*Donaldson & Lamont 2013*).

With no ATP being produced, the Na^+-K^+-ATPase pump is no longer functional. Cell walls then become permeable which can give rise to K^+ concentrations to levels that would be lethal during life. With the efflux of K^+, Na^+ accumulates inside the cell as a counter ion. On average, using concentrations after postmortem intervals ranging between 3 and 58 hours, serum Na^+ decreases from 140 to 120 mmol/L, while serum K^+ increases from 4 to 25 mmol/L (Singh et al. 2002).

The increase in intracellular Na^+ causes cell edema through osmosis, thereby progressively destroying cell membranes (*Yarema & Becker 2005*). Also, acidosis causes enzymatic digestion of cell membranes and leads to increased cell permeability (*Skopp 2010*). Changed membrane permeability can also lead to

leakage of albumin into tissues, thereby reducing the amount of albumin-bound drug in blood (*Ferner 2008*). Increased permeability of the intestinal wall enhances absorption of drugs from the gastrointestinal tract through passive diffusion (*Ferner 2008*).

Physicochemical properties affecting redistribution

There are several drug properties that might affect postmortem redistribution. One of the most discussed and researched properties is the volume of distribution (V_d). V_d is dependent on several other factors, including lipophilicity and ionization state of the compound but also age and gender. It is usually assumed that drugs with a V_d greater than 3 L/kg have the potential to undergo postmortem redistribution. However, this potential is influenced by the true distribution in the body, e.g. whether a compound accumulates in certain tissues. Ionization state becomes even more important with postmortem decreases in pH. As the body becomes more acidic, basic drugs will become (more) ionized, leading to higher levels of redistribution from tissues with high drug concentrations to tissues with low drug concentrations (*Yarema & Becker 2005*).

Other factors affecting redistribution

Postmortem redistribution might be influenced by several other factors. This paragraph lists a few of these. Firstly, environmental factors can play a role. The location and the position in which the body is found can influence redistribution. Temperature and humidity can have a significant effect on putrefaction, and thus the speed of decomposition. This causes an increase in microbial activity, and with that of drug degradation or neo-formation. Body position influences postmortem lividity or livor mortis, which is the settling of blood in the lowest part of the body, signified by a typical discoloration of the skin. Ongoing blood movement can be induced by transport of the deceased, resuscitation or by bloating of the stomach by microbial gas formation (*Yarema & Becker 2005*). Lastly, postmortem redistribution can be influenced by several personal factors such as gender, age, body mass index, body composition and underlying diseases.

Heart to femoral blood concentration ratio

As outlined in this chapter, postmortem drug redistribution should always be considered to avoid wrong interpretation of postmortem toxicological data. Postmortem redistribution mainly occurs from the central solid organs, thereby contaminating blood drug concentrations from centrally located

veins. Currently, to estimate the degree of postmortem redistribution, the forensic pathologist takes blood samples from a central location (heart blood) and a peripheral location (usually the femoral vein). The ratio of these blood drug concentrations is known as the central to peripheral blood concentration ratio (C:P ratio) or heart to femoral blood concentration ratio (HB:FB ratio).

To be able to compare HB:FB ratios between labs, all samples should be obtained in a similar way. Heart blood should preferably be collected from the right atrium of the heart, as the left side is more prone to redistribution as it is close to the trachea and lungs. Comparison of left and right heart blood may show large concentration differences. (*Yoshitome et al. 2008*) compared left and right heart blood concentrations of flecainide, an antiarrhythmic agent. Flecainide is a basic compound and accumulates in the lungs. Postmortem, redistribution occurs from the lungs resulting in high cardiac concentrations. As the left side of the heart is closer to the lungs, this resulted in threefold higher concentrations in left heart blood compared to right heart blood.

In addition, the collection of blood from a peripheral site can be difficult. The femoral vein is the preferred peripheral sampling site, as it is believed to be more representative of the antemortem blood concentration than heart blood. However, femoral blood concentrations are usually not equal to antemortem or perimortem blood concentrations (*Pélissier-Alicot et al. 2003*), leading to miscalculations of HB:FB ratios and misinterpretations of postmortem redistribution. In some cases, femoral blood concentrations were higher than heart blood concentrations, usually when resuscitation was employed (*Pélissier-Alicot et al. 2003*). When sampling blood from the femoral vein, it is important to avoid contamination from other veins. Contamination is most likely from blood originating in the inferior vena cava and the iliac vein, so these should be properly ligated before obtaining a blood sample (*Pélissier-Alicot et al. 2003*). Other sites have been discussed as a source of peripheral blood samples. An example of this is subclavian blood, which is usually considered as a peripheral site. Although recent research showed that subclavian blood concentrations were lower than heart blood concentrations, these concentrations were higher than femoral blood concentrations (*Molina & Hargrove 2013*). Subclavian blood should therefore be considered neither a central nor a peripheral site. This also shows the importance of documenting the sampling site to aid in the interpretation of obtained blood concentrations.

Compound class	Compounds tested	
Opiates/opioids	Acetylmorphine (B 9.1)	
	Codeine (B 9.2)	
	Methadone (B 9.1)	
	Morphine (B 9.1)	
	O-desmethyltramadol (B 9.0)	
	Propoxyphene (B 9.5)	
	Tramadol (B 9.2)	
Cocaine and its metabolites	Benzoylecgonine (B 9.5, A 3.2)	
	Cocaine (B 8.9)	
	Methylecgonine (B 9.0)	
Amphetamines	**Amphetamine** (B 10.0)	
	Methamphetamine (B 10.2)	
	3,4-methylenedioxyamphetamine (MDA) (B 10.0)	
	3,4-methylenedioxymethamphetamine (MDMA) (B 10.1)	
	3,4-methylenedioxy-N-ethylamphetamine (MDEA) (B 10.2)	
Cannabinoids	Tetrahydrocannabinol (THC) (N)	
	11-hydroxy-THC (11-OH-THC) (N)	
	11-nor-9-carboxy-THC (THC-COOH) (A 4.2)	
Benzodiazepines	1-Hydroxymidazolam (N)	Desmethylchlordiazepoxide (B 7.6)
	4-Hydroxyalprazolam (N)	**Diazepam** (N)
	4-Hydroxytriazolam (N)	Flunitrazepam (N)
	7-Acetamidoclonazepam (N)	Hydroxyethylflurazepam (B 8.2)
	7-Acetamidonitrazepam (N)	Lorazepam (N)
	7-Aminoclonazepam (N)	Lormetazepam (N)
	7-Aminoflunitrazepam (N)	Midazolam (N, but B 6.6)
	7-Aminonitrazepam	N-desalkylflurazepam (N)
	Alprazolam (N)	N-desmethylclobazam (N)
	Bromazepam (N)	N-desmethyldiazepam (N)
	Brotizolam (N)	N-desmethylflunitrazepam (N)
	Chlordiazepoxide (N, but B 6.4)	Nitrazepam (N)
	Clobazam (N)	**Oxazepam** (N)
	Clonazepam (N)	**Temazepam** (N)
	Demoxepam (A 2.8)	Triazolam (N)
Other hypnotic drugs	Zolpidem (N, but B 5.6)	
	Zopiclone (N, but B 6.9)	
Antidepressants	**Amitriptyline** (B 9.8)	
	Citalopram (B 9.8)	
	Fluoxetine (B 9.8)	
	Fluvoxamine (B 9.2)	
	Nortriptyline (B 10.5)	
	Paroxetine (B 9.8)	
Other	Lidocaine (B 7.8)	

Table 1. Drugs included in the targeted screening at the Netherlands Forensic Institute. List of 57 drugs of abuse which are always included in the analysis of bodily fluids after autopsy at the Netherlands Forensic Institute. Compounds in bold are studied in this thesis, along with other compounds which were selected based on their physicochemical properties alone. pKa values are given if a compound is ionized at physiological pH (B for base moiety, A for acid moiety, N for compounds that are not substantially ionized between pH 4 and 8). pKa values were obtained from (*ChemAxon, www.chemicalize.org*).

The main downfall of the HB:FB ratio is the lack of a time component. Blood samples are usually collected during autopsy, but the interval between time of death and forensic autopsy can be long, which limits the use of HB:FB ratios without mentioning the postmortem time interval. Another factor that is overlooked in the HB:FB ratio, is the cause of death. It would seem logical that redistribution phenomena could be different for deaths caused by e.g. drug overdose, drowning or trauma. To help the interpretation of postmortem toxicological results, it is not only of importance to compare data from different sampling sites, but also to include data taken at different postmortem time intervals. These time-dependent studies are necessary to establish the rate of change in drug concentrations, and could ultimately help to determine relevant conditions that influence postmortem redistribution.

ANALYSIS IN FORENSIC STUDIES

Compounds of interest

In forensic studies, analysis of blood samples that potentially contain unknown substances is based on both general and targeted screening. The targeted screening usually focusses on the most commonly used drugs of abuse (DOA). At the Netherlands Forensic Institute, this DOA run comprises 57 different compounds. These are listed in table 1, where the compounds that are used in this thesis are printed in bold. For the DOA set of 57 compounds, 29 compounds (51%) are not ionized at (neutral) physiological pH, while 26 compounds (47%) contain basic groups that are predominantly charged at neutral pH, and 4 compounds (7%) have largely dissociated acidic groups at neutral pH.

The test compounds used in this thesis were selected based on their occurrence in table 1 and their physicochemical properties. Important drug

properties included pKa (as we mainly focused on ionizable compounds which are present as cation at physiological pH), lipophilicity (as depicted by log K_{ow}) and the amount of plasma protein binding. The aim was to establish a set of test compounds that represented different compound classes, with a variation in pKa, log K_{ow} and plasma protein binding.

Postmortem specimens and extraction of chemicals

Postmortem specimen collection and analysis can differ from antemortem specimens. The choice of specimen in forensic toxicology may be case dependent, as not all specimens may be available or useful for interpretation. The most commonly used specimens are blood and urine, but other specimens can contribute to a correct interpretation of postmortem obtained results.

Blood

In postmortem drug analysis, whole blood is used, as separation of red blood cells from serum is usually not possible (*Skopp 2010*). This already poses some problems as antemortem reference values are usually determined in plasma, thereby neglecting the fraction bound to red blood cells. To estimate concentrations in whole blood from plasma concentrations, the blood-to-plasma concentration ratio can be used (*Uchimura et al. 2010*). Antemortem, blood concentrations may differ when sampling arterial blood or venous blood. During absorption, arterial blood concentrations are higher than venous blood concentrations, while the opposite is true during the elimination phase (*Pélissier-Alicot et al. 2003*). Postmortem, blood concentrations from central parts of the body may be higher than those taken from peripheral sites. This is the result of postmortem redistribution from central organs (*Kennedy 2010*). To estimate the degree of postmortem redistribution, blood samples are collected from both a central and a peripheral site. The HB:FB ratio can be used to estimate redistribution processes. The work-up of blood may include simple protein precipitation using acetone, followed by several centrifugation steps and a dilution with ultrapure water. In specific cases, extraction techniques such as solid-phase extraction (SPE) are used to increase sensitivity. For SPE, the blood sample is mixed with buffer, vortexed and centrifuged and passed over an SPE column. The compounds can be eluted in one or more fractions, for instance a fraction containing neutral and acidic drugs and a fraction containing basic drugs. The organic phase in the eluates is evaporated and the compounds are reconstituted in acetonitrile.

Urine

Urine can be a useful tool for screening purposes, as most compounds are excreted via the urine (*Kennedy 2010*). However, urine can only be used to determine the presence of a compound, as drug concentrations are dependent on urine production. Urine is analyzed after a simple work-up that includes protein precipitation using acetone, centrifugation and a dilution. Due to the presence of conjugated drug metabolites, urine samples need to be deconjugated before analysis. Again, in specific cases, SPE can be used to increase sensitivity.

Vitreous humor

Vitreous humor is the gel-like fluid in the eye, between the lens and the retina, and is essential in maintaining the spherical shape of the eye. Drug concentrations in vitreous humor are reportedly comparable to drug concentrations in the blood. Vitreous humor can be easily obtained postmortem and is not quickly contaminated by microorganisms (*Coe 1993*). As the vitreous humor contains very little proteins, extraction of drugs is relatively easy (*Drummer & Gerostamoulos 2002*). Like urine and blood, vitreous humor can also be analyzed after protein precipitation and a dilution step. The downside of using vitreous humor is that antemortem reference concentrations in human vitreous humor are not available, as removal of the vitreous humor by life is not possible.

Liver

As the liver is a drug reservoir, direct sampling of liver tissue can be useful. As some parts of the liver are close to the intestine, it is recommended to take a sample from deep inside the right lobe (*Drummer & Gerostamoulos 2002*). A recent study suggested to use the ratio between drug concentrations in liver and blood to estimate postmortem redistribution (*McIntyre 2013*). A ratio below 5 would indicate no redistribution occurred, while a ratio above 20 or 30 would mean the drug has a high tendency to be redistributed. However, this leaves quite a large grey area where interpretation of the results remains difficult. To be able to analyze tissue, it can be homogenized with water using a turrax. This solution can then be simply diluted after protein precipitation and analyzed, or first extracted by using SPE.

Hair and nails

Hair and nails can be used to determine chronic exposure, as it can take days to weeks before the drug is detectable in these specimens. Drugs can be detected up to months or years after exposure, depending on the length of the hairs or

nails (*Drummer & Gerostamoulos 2002*). A downfall of the use of these type of specimens is that these can also be contaminated by an external source. The drug/metabolite ratio could then be an indication that the drug was actively consumed. Before analysis, hairs and nails should be washed to reduce outside contamination. After this, the hair is extracted using highly acidic, highly basic or organic solvents for several hours, usually employing some heating of the sample. The resulting sample can be analyzed directly or transferred to a SPE column for further extraction.

Muscle or fat tissue

Muscle and fat tissue make up a large portion of the human body, representing a large fraction of the total body burden. However, tissue concentrations can be low and the extraction processes needed prior to analysis to extract the drug from the tissue can be unreliable. Moreover, due to differences in the blood supply, there can be high variability in concentrations within the tissue (*Drummer & Gerostamoulos 2002*). The clean-up and extraction of muscle and fat tissue is similar to the work-up of liver tissue.

Studying postmortem redistribution

This section will discuss a research paper on the postmortem redistribution of Δ^9-tetrahydrocannabinol (THC), the active ingredient in cannabis, in pigs (*Brunet et al. 2010*). Focus here is on the experimental part of this research, not on its results or the interpretation thereof.

Fifteen Large White male pigs (29-50 kg) received 200 µg/kg bodyweight of THC through an intrajugular injection. Two hours after THC administration, all pigs were euthanized by KCl injection. Autopsies were performed at 0, 6, 15, 24 and 48 hours, with three pigs autopsied at each postmortem time interval. Concentrations in different organs at all postmortem intervals were related to the concentrations in these organs of the first three pigs analyzed, which were set at 100% (figure 3).

Relative concentrations found (mean of three pigs) decrease in some tissues (e.g. muscle), but increase in other tissues (e.g. brain). However, all determined concentrations show very high standard deviations, as shown in figure 3 for the kidney (right). As the results of three pigs are pooled, this is most likely due to interindividual differences. ADME processes antemortem may differ between pigs, leading to different concentrations at t=0. Moreover, several individual factors influence postmortem redistribution, thereby contributing to the high

variations in determined concentrations at all postmortem intervals.
To eliminate these interindividual differences, the same animal should have been sampled at each time point. However, in obtaining the specimens needed for analysis, the existing equilibrium between tissue and blood concentrations will be disturbed after sampling. For instance, when removing 50 mL of blood from the heart, this will create a concentration gradient in the heart which will give rise to changed diffusion processes as would otherwise occur in regular postmortem redistribution.

So, to be able to perform time-dependent studies on postmortem drug redistribution, a sampling tool is needed that is fast, sensitive and employable in all types of specimens (both blood and tissue), and does not disturb the existing equilibria or kinetic processes occurring in the postmortem situation. This thesis describes the development and validation of solid-phase microextraction as sampling tool for forensic applications.

SOLID-PHASE MICROEXTRACTION (SPME)

In any analytical procedure, sample preparation is an essential but time-consuming process. To address this, solid-phase microextraction (SPME) was developed in the early 1990s as a faster alternative to the existing extraction techniques (*Arthur & Pawliszyn 1990*). As in the name, SPME is a microextraction technique, employing a very small amount of extraction material, thereby not necessarily aiming for an exhaustive extraction but for an equilibrium extraction. This means that by using SPME, in certain conditions repeated sampling is possible, making it an ideal tool to study postmortem redistribution.

General principle of SPME

The SPME fiber consists of a glass or metal core with a polymer coating that is immersed in the matrix containing the analyte (figure 4). In samples containing binding matrix (e.g. plasma proteins), only the free fraction of the analyte is taken up by the fiber. Microextraction is complete when equilibrium is reached between fiber concentration and sample concentration. Fiber concentration is then proportional to sample concentration (*Pawliszyn 2012*).

Sampling from a closed system follows the law of conservation of mass:

$$C_0 \cdot V_s = C_s^{\infty} \cdot V_s + C_f^{\infty} \cdot V_f \quad \text{(Eq. 1)}$$

Where the initial mass in the sample ($C_0 \cdot V_s$) is divided over two phases after equilibrium exposure, i.e. as the remaining mass in the sample ($C_s^{\infty} \cdot V_s$) and the mass sorbed to the fiber ($C_f^{\infty} \cdot V_f$).

The fiber/sample partition coefficient K_{fs} is the affinity of an analyte to the SPME fiber and is defined as the ratio between fiber and sample concentration:

$$K_{fs} = \frac{C_f^{\infty}}{C_s^{\infty}} \quad \text{(Eq. 2)}$$

Typically, K_{fs} is given as its log value log K_{fs}:

$$\log K_{fs} = \log C_f^{\infty} - \log C_s \quad \text{(Eq. 2a)}$$

The combination of equation 1 and 2 results in:

$$C_f^{\infty} = C_o \cdot \frac{K_{fs} K_{fs}}{K_{fs} \cdot V_f + V_s} \quad \text{(Eq. 3)}$$

With large, but unknown, sample volumes (e.g. air, water, blood), the sample volume is several magnitudes bigger than the fiber volume. In that case, $V_s >> K_{fs} \cdot V_f$ and equation 3 becomes:

$$C_f^{\infty} = K_{fs} \cdot C_o \quad \text{(Eq. 4)}$$

This allows for the estimation of the initial concentration when K_{fs} is known (after calibration). The partition coefficient K_{fs} is not only dependent on the analyte of interest, but also dependent on the type of SPME coating used and certain properties of the sample matrix, such as pH and ionic strength. In this thesis, partition coefficients that are determined in specific circumstances (e.g. at a single pH) are termed the fiber/water distribution coefficient or D_{fw}.

The initial concentration in the sample (C_0) from equation 4 can only be used when no depletion of this concentration occurs during extraction (*Lord & Bojko 2012*). If the amount extracted from the sample decreases the sample concentration to such an extent that it is significantly different from the initial concentration, this is called depletion. When the amount extracted is negligible,

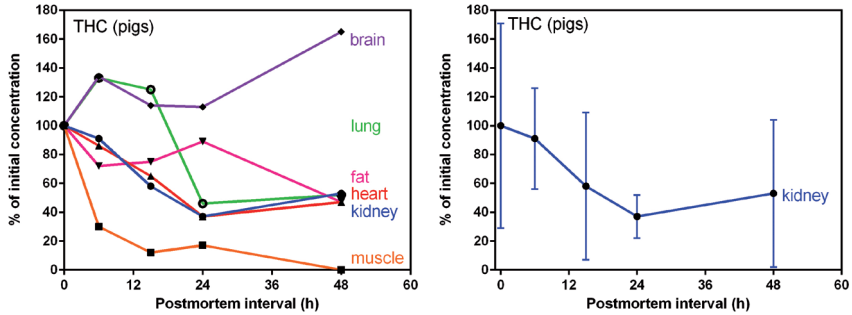

Figure 3. Relative changes in postmortem tissue concentrations of THC in pigs. Left: mean tissue concentrations in different tissues at various postmortem time intervals. Right: tissue concentration in the kidney, plotted as mean ± standard deviation. Adapted from (*Brunet et al., 2010*).

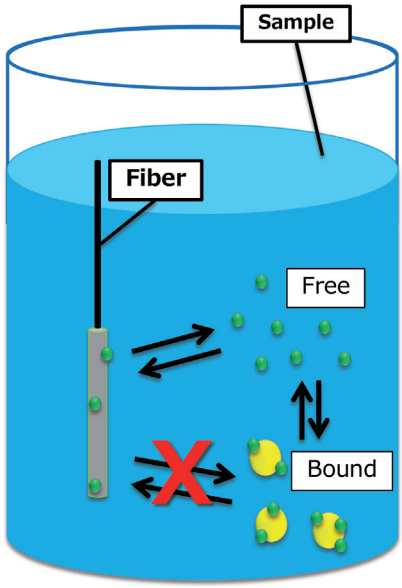

Figure 4. Exposure of a SPME fiber to a sample leads to uptake of the free concentration until equilibrium is reached. The bound concentration is not taken up by the fiber.

that is the amount extracted is less than 5%, the initial concentration can be used to calculate partitioning (*Vaes et al. 1996*). This is of specific interest in a system where a compound is present as freely dissolved and bound to a certain matrix (for instance proteins). Negligible depletion SPME will then ensure that the partitioning equilibrium between other phases is not disturbed, such as blood/tissue partitioning or free vs. protein-bound equilibrium (*Heringa & Hermens 2003*). However, if the desorption of a chemical bound to the matrix back into the aqueous phase is fast (labile complex), depletion is allowed as long as the amount that is bound is not affected by the fiber extraction. This is called matrix SPME (*Mayer et al. 2000*). The strength of negligible depletion SPME is that it allows for repeated sampling in the same system.

Sampling of ionized compounds

When SPME was first developed, the coatings available were compatible with hydrophobic, neutral compounds. However, many analytes of interest contain ionizable groups, clearly showing a need for sampling tools that are able to extract charged compounds. In this thesis, the focus is on pharmaceuticals and illicit drugs, as these are most commonly seen in forensic cases. Moreover, the emphasis is almost exclusively on bases, which are more likely to undergo postmortem redistribution, as previously discussed.

Ionizable compounds

Ionizable compounds become ionized based on their pKa and the pH of the sample. This relationship is given by the Henderson-Hasselbalch equation:

$$pH = pKa + \log\left(\frac{[\text{unionized}]}{[\text{ionized}]}\right) \quad \text{(Eq. 5)}$$

In an unbuffered system, the degree of ionization of a compound determines the pH of the solution. In a buffered system, e.g. in blood and as used in the experimental work in this thesis, the pH will determine whether a compound is predominantly present in its neutral or charged form. Figure 5 shows the degree of ionization at different pH, for three different pKa values. From this graph and the Henderson-Hasselbalch equation, it can be deduced that when the pH equals the pKa, 50% of the compound's molecules are charged (and thus 50% are present as neutral species). For basic compounds, when the pH is one value below the pKa of the compound, the compound will be 90% charged. When the pH is two values below the pKa, the compound is 99% charged.

It is estimated that around 65% of all pharmaceuticals are ionizable, of which 50% contain only cationic groups (*Manallack 2009*). Roughly 70% of these bases have a pKa between 6 and 10 (*Manallack 2009*), making them (partially) charged at physiologically relevant pH.

Neutral SPME coatings

The most commonly used SPME coatings include polyacrylate (PA), polydimethylsiloxane (PDMS) and polyethyleneglycol (PEG) (*Shirey 2012*). These coatings are neutral coatings, i.e. they are only capable of hydrophobic interactions. An exception to this is polyacrylate, which contains anionic groups at pH>5 (*Chen et al. 2012*). For these neutral SPME coatings, the analysis of ionized compounds is only possible based on the available neutral fraction.

Early studies on ionizable compounds using SPME therefore manipulated the sample matrix before extraction, for example for the extraction of lidocaine from human plasma (*Koster et al. 2000*). By changing the pH, the extraction efficiency of the SPME method could be increased as the neutral fraction of the compound is increased. However, manipulation of the sample matrix might not always be desirable or possible. Examples of this are *in vivo* measurements, where matrix modification is not possible, or the assessment of drug protein binding, where altering the pH of the sample will also alter the protein binding in itself.

C18/SCX fiber

To study ionizable compounds using SPME, a prototype fiber was developed by Supelco. This fiber consists of a biocompatible nitinol support wire coated with two different sorption phases. This "mixed-mode" coating consists of silica particles with both C18 chains and propylsulfonic acid groups (figure 6). The fiber is capable of extracting hydrophobic, neutral compounds through interaction with the C18 chains but also polar, positively charged chemicals through interactions with the propylsulfonic acid groups, which serve as strong cation exchange sites.

Only a few research papers have addressed the use of mixed-mode SPME fibers. The C18/SCX fiber was first described in a comparison of 42 different SPME coatings (*Vuckovic & Pawliszyn 2011)*. Here, the authors evaluated the sensitivity of these SPME coatings to simultaneously extract 36 different metabolites, ranging in log K_{ow} between -7 and 7. The C18/SCX fiber was among the three fibers that performed the best. This fiber was subsequently used to

study the metabolome of mice in vivo, by placing the fiber directly in blood through an external catheter located in the common carotid artery (*Vuckovic et al. 2011*). Using this specific SPME fiber, metabolites could be found that cannot be detected using conventional methods, including metabolites with a high metabolic turnover.

Extraction parameters influencing sorption

There are certain extraction parameters that can influence sorption to the fiber. Most of these are related to solution chemistry and include pH, temperature and ionic strength. pH is likely to influence sorption, as it determines whether an ionizable compound is present in its neutral or charged form. The neutral and charged form can have different sorption affinities for SPME fibers, making pH an important factor for some compounds. Another factor influencing sorption is temperature. Increasing the temperature of the sample during extraction will increase uptake kinetics of the compound to the fiber, as the rate of diffusion is increased. Conversely, the sorption affinity of the compound will decrease, due to an increase in solubility (*Pawliszyn 2012*). For some compounds, increasing the temperature will increase their volatility, which increases extraction efficiency when using headspace analysis but causes a loss of compound in the aqueous phase, thereby negatively influencing direct immersion SPME. The ionic strength of the solution can have different effects on sorption affinity. For neutral compounds, adding large amounts of salts can benefit sorption (so-called salting-out effect). For ionized compounds, ions can enhance sorption through ion pairing (with Na^+ or Cl^-) or calcium-bridging (for anionic compounds) (*Haftka et al. 2015*). However, ions can also decrease sorption, when sorption is mediated by electrostatic interactions, as is the case for ionized compounds to charged SPME coatings. Another factor to take into account is agitation of the sample. Agitation decreases the time needed to reach equilibrium, as the mass transfer to the fiber is increased (*Kudlejova et al. 2012*). Sorption affinity at equilibrium is not influenced by the degree of agitation, but for many biomedical applications, extremely short sampling is required. In those cases, agitation can help to reach the needed sensitivity in such a short time interval.

Absorption vs. adsorption

SPME coatings can either extract analytes based on absorption or adsorption. In absorption, the analyte diffuses through the liquid to reach the outside of the fiber, where it diffuses further into the bulk of the fiber coating. As the analyte "dissolves" in the fiber coating, the relationship between the concentration in the sample and in the fiber coating can be described using a

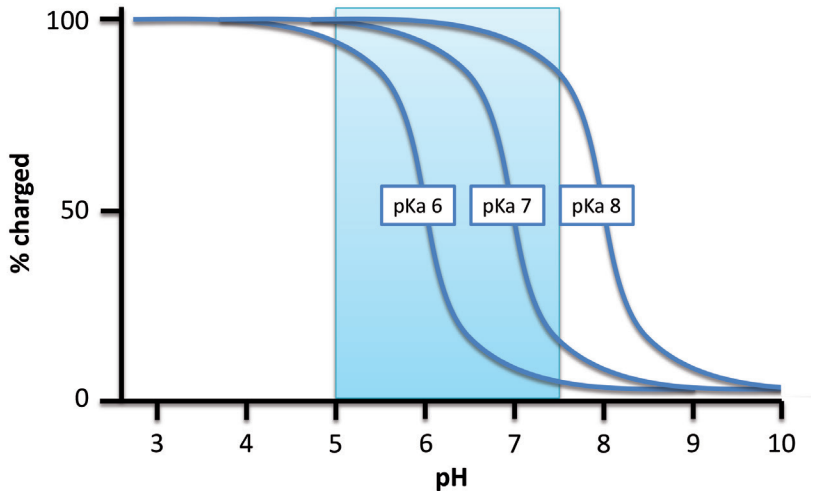

Figure 5. Charge state of basic chemicals with different pKa. When pH = pKa, the compound is 50% present as neutral species and 50% as charged species. The box indicates the physiologically relevant pH range (5.0 – 7.4).

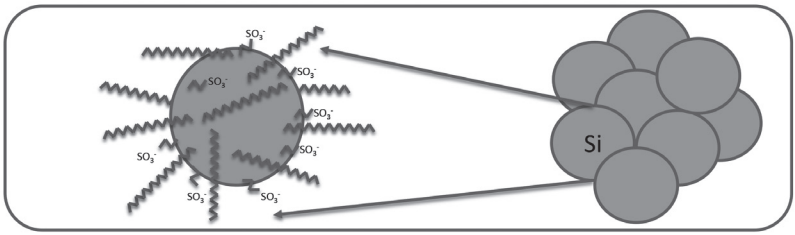

Figure 6. Schematic representation of the coating of the C18/SCX fiber. Both C18 aliphatic carbon chains and propylsulfonic acid groups are bound to silica particles. These silica particles are then bound to the support wire using a polymeric binder. C18/SCX fibers are prototype SPME fibers from Supelco.

linear absorption isotherm:

$$C_f = K_{fs} \cdot C_s \qquad \text{(Eq. 6)}$$

Where C_f and C_s are fiber concentration and sample concentration, respectively, and Kfs is the partition coefficient between these concentrations.

Contrary to absorption, where the analyte dissolves in the fiber coating, the analyte can also bind to the surface of the coating. This is called adsorption. In case of an adsorption process, a nonlinear Freundlich isotherm is often applied:

$$C_f = K_F \cdot C_s^{n_F} \qquad \text{(Eq. 7)}$$

Here, K_F and n_F represent the Freundlich constant and Freundlich exponent. When concentrations are logarithmically transformed, equation 7 becomes:

$$\log C_f = \log K_F + n_F \cdot \log C_s \qquad \text{(Eq. 7a)}$$

When plotting data in a graph, the exponent nF represents the slope of the Freundlich fit. This provides direct information about the linearity of the relationship between fiber and sample concentration. A Freundlich slope of 1 indicates a completely linear relationship. For sorption of analytes to SPME fibers, usually Freundlich slopes are below 1, as sorption decreases at higher concentrations.
The mathematical description of the Freundlich equation is based on providing a good fit for empirical data, but the model is not based on a theoretical assumption of analyte behavior in solution (*Górecki 1999*). One model that is based on a theoretical mechanism is the Langmuir equation, which is often used to describe adsorption processes. As the analyte sorbs only to the surface of the coating, adsorption is by definition a competitive process, as there are limited sorption sites available. This will result in linear sorption at low concentrations where only a few sorption sites are occupied, after which sorption levels off to a maximum. This is mathematically described in the Langmuir equation:

$$C_f = \frac{C_{f,max} \cdot K_L \cdot C_s}{1 + K_L \cdot C_s} \qquad \text{(Eq. 8)}$$

Here, the relationship between the fiber concentration C_f and the sample concentration C_s is defined by the maximum sorption capacity ($C_{f,max}$) and a Langmuir constant K_L. A limitation of the Langmuir equation is that the fit is linear up to 10% of $C_{f,max}$. This model alone cannot account for empirical data with a less than linear fit, or when data levels off below 10% of $C_{f,max}$.

The SPME coating used in this thesis, the C18/SCX fiber, employs a combination of two extraction phases. As sorption to the fiber is governed by (at least) two different sorption mechanisms, analytes can sorb with different chemical interactions due to their charge, but also the presence of other polar and nonpolar structural moieties. Fitting sorption data with the above described models is therefore not as straightforward as with neutral compounds and simple coatings such as PDMS or analogous C18. Obviously, multiple other models exist that can then be employed to fit the data. These models include dual mode isotherms, which are combinations of linear, Freundlich and Langmuir equations (*Schwarzenbach et al. 2003*). However, any model should be realistic, predictive, effective and comprehensive (*Limousin et al. 2007*), which limits the use of complex models in sorption processes that are mediated by unknown mechanisms.

SCOPE OF THIS THESIS

The main focus of this thesis is the sampling of ionized compounds using the C18/SCX fiber. The development and validation of a useful SPME method to study postmortem redistribution is the major objective, as highlighted in this Chapter (Chapter 1).

This thesis is divided into two parts. Part I focuses on the sorption of ionized chemicals to the C18/SCX fiber, and provides a mechanistic insight into the extraction process and which parameters influence fiber uptake. Chapter 2 compares the C18/SCX fiber with other, more conventional, SPME coatings. Amphetamine is used as a model ionizable drug of abuse to show the influence of pH and ionic strength on sorption to different passive sampler coatings. This chapter also discusses the saturation concentration of the C18/SCX fiber in relation to the "therapeutic-toxic-lethal" concentrations in blood, which is further studied in the subsequent chapter. Chapter 3 expands the research on amphetamine, but also includes the more hydrophobic cation amitriptyline. Here, changes in electrolyte homeostasis (as they might occur postmortem) were simulated to determine its effect on sorption affinity. Competition effects were tested by using binary mixtures of amphetamine and amitriptyline. Part I concludes with a collection of sorption data of ionized chemicals to the C18/SCX fiber (Chapter 4). This chapter shows data for cationic compounds with relative simple molecular structures, and tries to find common denominators that define sorption affinity. This is then extended to a set of thirty ionized pharmaceuticals.

Part II of this thesis shows the use of the C18/SCX fiber as sampling tool in two different biomedical applications. In Chapter 5, the C18/SCX fiber is used to measure freely dissolved concentrations of amitriptyline, amphetamine, diazepam and tramadol. Binding matrices varied from albumin to plasma and whole blood. The unwanted binding of protein to the fiber, known as fouling, is extensively addressed. Chapter 6 discusses the potential of C18/SCX fibers as a direct sampling tool in unhomogenized tissue. First, agarose gel was used as a tissue surrogate to study uptake and sorption affinity of diazepam and amitriptyline. Consecutively, pork muscle tissue was loaded with these compounds and SPME fibers were directly inserted into the tissue to test the applicability of the C18/SCX fiber as a direct tissue sampler.

Chapter 7 provides a general discussion of the experimental chapters. First, the experimental work on sorption of cationic compounds to the C18/SCX fiber is discussed. This is followed by the introduction of a model to predict *in vivo* distribution of cationic compounds. The next section discusses the experimental work on the application of the C18/SCX fiber as sampling tool to study different sorption processes. Lastly, the potential of SPME as sampling tool in forensic applications is discussed.

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ELUCIDATING THE SORPTION MECHANISM OF "MIXED-MODE" SPME USING THE BASIC DRUG AMPHETAMINE AS A MODEL COMPOUND

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ABSTRACT

We studied the sorption of amphetamine as a model drug to represent small, polar organic cations to a new SPME coating combining C18 and propylsulfonic acid. This combination of hydrophobic and strong cation exchange (SCX) groups was compared to conventional SPME fibers with polyacrylate (PA) or C18 coating. The affinity of amphetamine at physiological pH (PBS) was 20 to 180 times greater for the new C18/SCX coating than for C18 alone and PA of different coating thickness. As amphetamine is a base and >99% protonated at physiological pH, this enhanced affinity is attributed to the ion-exchange phase in the coating. Tests at pH above the pKa of amphetamine show that, when normalized to the coating volume, neutral amphetamine also has a higher affinity compared to PA. As ion-exchange groups are not unlimitedly present in the coating, amphetamine isotherms level off to a saturation concentration on the C18/SCX fiber at the highest tested aqueous concentrations. Also, other cations (Na^+, K^+, Ca^{2+}) compete for the SCX sites and decrease the sorption coefficients, e.g. by 1.7 log units when comparing Milli-Q water with PBS. The C18/SCX fiber provides improved sensitivity over some of the classic SPME fibers. However, care should be taken near the cation exchange capacity of the fiber and the fiber should be calibrated in an appropriate matrix so as to eliminate competition effects.

INTRODUCTION

Solid-phase microextraction (SPME) was developed in the early 1990s as a simple extraction technique with several advantages over the conventional sampling methods (*Arthur & Pawliszyn 1990*), including decreased sampling time, decreased use of solvents and improvement of detection limits (*Kataoka 2003*). When SPME was first developed, the coatings available were applicable to hydrophobic, neutral compounds (*Buchholz & Pawliszyn 1994, Lord & Pawliszyn 2000, Pawliszyn 2012, Theodoridis et al. 2000*). Recently, newer coatings have become available, making it possible to extract more polar and charged molecules (*Vuckovic et al. 2009*).

The analysis of pharmaceuticals using SPME started soon after its discovery. SPME was first applied as a headspace sampling device to determine volatile compounds in plasma and urine samples (*Alizadeh et al. 2008, Lai et al. 2008, Lee et al. 2000, Namera et al. 2000, Okajima et al. 2001, Raikos et al. 2009, Sha et al. 2005, Tsoukali et al. 2004*). *In vivo* sampling of pharmaceuticals was made possible by using biocompatible fibers and extracting drugs that are neutral at physiological pH such as diazepam (*Lord et al. 2003, Musteata et al. 2006*) and carbamazepine (*Vuckovic et al. 2011b*). However, many pharmaceuticals are basic compounds and protonated at physiological pH and in many studies, it is desirable not to disturb the system conditions during extraction. Consequently, *in vivo* SPME of these drugs is usually based on their small neutral fraction (*Togunde et al. 2012*). Until recently, it was presumed that SPME coatings only extract the neutral species of analytes (*Risticevic et al. 2010*). This indicates there is a need for coatings that can extract the large fraction of protonated species, because this may increase the sensitivity of the analysis of these cationic pharmaceuticals. Extraction of organic cations has been previously addressed through (1) restricted-access SPME (*Walles et al. 2004*), where not only the inner volume, but also the outer surface possesses sorptive properties (*Chiap et al. 2002*), thereby reducing the selectivity of the fiber, (2) electrochemically enhanced SPME (*Zeng et al. 2011*), which is not biocompatible *in vivo* due to the large electrode used to increase sorption, and (3) by exploring the ion-exchange capability of polyacrylate fibers (*Chen et al. 2012*).

While partitioning of neutral chemicals to classic SPME coatings such as (PDMS) is mainly driven by hydrophobicity (*Endo et al. 2011, Sprunger et al. 2007*), the interactions of polar and ionized chemicals with the newer types

of coatings can be much more specific. Recently, a large study (*Vuckovic & Pawliszyn 2011*) investigated the capacity to extract a variety of metabolites simultaneously for different SPME coatings, mostly based on conventional solid phase extraction material, including ion-exchange coatings and prototype combinations with hydrophobic phases (so-called mixed-mode coatings). One of these mixed-mode coatings, consisting of C18 and benzenesulfonic acid (strong cation exchange; SCX), showed the best performance and was applied in an untargeted metabolomics profiling study (*Vuckovic & Pawliszyn 2011*). This mixed-mode coating was subsequently applied in a metabolomics study using mice dosed with carbamazepine, where the ability of this coating to extract a wide range of metabolites *in vivo* was confirmed (*Vuckovic et al. 2011a*). Coatings with ion-exchange capacity may especially provide relatively high yields for small (polar) organic cations, for which the small neutral fraction has insufficient affinity to sorb to classic SPME coatings. However, the adsorption processes for ionic compounds may strongly depend on the pH of the solution, as well as concentrations of other cations such as Na^+ and Ca^{2+} because they may compete for ion-exchange binding sites on the SPME coating (*Chen et al. 2012, Droge & Goss 2012, Karthikeyan & Chorover 2002, Sibley & Pedersen 2008*).

The final goal for our work is to develop SPME methods that are applicable in *in vivo* and in forensic studies to detect and quantify several pharmaceutical and illicit drugs in a wide (therapeutic) concentration range. In this study, we use amphetamine as a small, basic model drug (pKa 9.9 (*Baselt 2004*)) to test the applicability of the prototype combination of C18 and propylsulfonic acid (C18/SCX) as coating material compared to the conventional SPME coating of polyacrylate. We aim to get more mechanistic insight into the sorption mechanism of amphetamine to the various SPME fibers by exploring the influence of amphetamine concentration, the pH and the presence and concentration of electrolytes on sorption to the fiber. We have chosen physiological conditions for the exposure solution to emphasize the capability of the fiber to extract amphetamine in physiological circumstances.

MATERIALS AND METHOD

Chemicals and materials

Mixed-mode fibers containing silica particles, bonded with C18/propylsulfonic acid, were kindly provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). These fibers consisted of 3 cm pieces of nitinol wire (202 µm diameter) with

a 1.5 cm coating length at an average thickness of 45 µm. The coating was prepared with HPLC column grade silica based material bound on the wire with a biocompatible polymeric binder. The silica particle has a mean pore size of 100 Å. The mean particle size is 3 µm and the total surface area is 450 m^2/g. The target sulfur loading was reported to be 2-2.5%. In this study, the prototype fibers were re-used after cleaning with desorption fluid, although they are intended to be single use products. Consistency for several prototype batches and performance after repeated use was monitored, as outlined below. Fibers with a silica core (108 µm diameter) coated with polyacrylate (7.5 and 34.5 µm coating thickness) were obtained from Polymicro Technologies (Phoenix, AZ, USA). SPME-LC fiber probes (C18 functional group, 45 µm coating thickness, 1.5 cm coating length) were from Supelco, Sigma Aldrich and consisted of a fiber (200 µm diameter support) within a hypodermic needle. Amphetamine hydrochloride was purchased from Spruyt Hillen (IJsselstein, The Netherlands). Phosphate-buffered saline (PBS) consisted of 138 mM NaCl, 8 mM of Na_2HPO_4, 1.5 mM of KH_2PO_4 (all Merck, Darmstadt, Germany), and 2.7 mM KCl (Sigma Aldrich, Zwijndrecht, The Netherlands) dissolved in Milli-Q water (18.2 MΩ•cm, Millipore, Amsterdam, The Netherlands). $CaCl_2$ was obtained from Sigma Aldrich. Ammonia solution (25%) used was from Merck. Methanol and acetonitrile were HPLC-grade (BioSolve, Valkenswaard, The Netherlands).

SPME procedures with C18/SCX fibers

Sorption experiments were performed in HPLC autosampler vials (Grace, Breda, The Netherlands) with a volume of 1.5 mL. Different amphetamine concentrations were made by diluting a stock solution in methanol of 7.4 mM amphetamine to aqueous concentrations ranging between 0.74 and 74 µM. All working solutions had a methanol content of <1%. C18/SCX fibers were exposed to a range of concentrations and exposure times. Two hours was chosen as standard exposure time for experiments at equilibrium, with the exception of the sorption experiment at pH ranging between 2 and 12 where exposure time was 4 hours. Vials were placed on a Stuart Roller Mixer SRT9D at 60 rpm during exposure. As a result of the current fiber set-up, faster agitation of the fibers might damage the coating and was therefore not applied. After a certain exposure time, fibers were transferred to desorption vials, HPLC autosampler vials with a 200 µL insert containing 120 µL of desorption fluid. The desorption fluid consists of 90% acetonitrile and 10% Milli-Q water with 10% NH_3 in the water phase, resulting in an end concentration NH_3 of 1%. The pH of this solution is approximately 11 to make sure amphetamine

is mostly neutral and can be captured by the relatively large volume of the organic phase in comparison with the small fiber coating volume. The fibers were desorbed statically for at least 15 minutes, after which they were removed. The high pH of the desorption fluid made it necessary to acidify the samples before injection into the HPLC as the injected solution should approximate the mobile phase. The 120 µL desorption fluid used to desorb the fibers was therefore acidified with 60 µL of 0.1 M HCl and vortexed. Although we intended to arrive at a pH of 3, the actual pH was higher. This may have affected the HPLC column, however, we tested the desorption with a lower NH_3 concentration which could be sufficiently acidified and found it did not affect the results. This and the time needed to fully desorb amphetamine from the mixed mode fiber are described in the Supporting Information, Figure S1. Since the fibers were re-used for several experiments, they were pooled after each experiment and left in desorption fluid overnight. After this, they were stored in 1:1 (v/v) methanol:PBS until the next experiment. To confirm no amphetamine had remained on the fiber since the last experiment, every experiment incorporated fiber blanks in triplicate from exposure solutions that were not spiked with amphetamine. Furthermore, to ensure the quality of the fibers remained equal over several experiments, repeat experiments were regularly performed to determine whether a slow decrease in sorption capacity occurred. If this was the case, the fibers were discarded and new fibers were used for the next experiments. These were also first checked to determine whether their sorption capacity was comparable to the previous set of fibers.

SPME procedures with PA and C18 fibers

PA fibers (both 7 and 35 µm) were cut to lengths of 2 – 4.5 cm (±0.2 mm) using a guillotine paper cutter. The fibers were temperature-conditioned overnight (16 hours) in the oven of a gas chromatograph under nitrogen flow at 120°C and stored >24 hours in Milli-Q water before conducting sorption experiments, following previous work with ionic compounds (*Chen et al. 2012, Rico-Rico et al. 2009*). Volume of exposure solutions was 5 mL with exception of the pH experiment where the volume was 20 mL. Standard equilibrium exposure time for the PA fibers was 24 hours. Vials were placed on a Stuart Roller Mixer SRT9 at 40 rpm during exposure. The fibers were desorbed in autosampler vials with insert using 125 µL 12% acetonitrile in a 10 mM phosphate buffer at pH 3 for at least 23 hours.

C18 fibers were preconditioned as instructed by the manufacturer. The needles were then inserted through the vial septum of 1.5 mL HPLC autosampler vials and the fibers exposed to different concentrations of amphetamine. Exposure solution was a 10 mM phosphate buffer, pH 7.4, or a 10 mM carbonate buffer, pH 9.0. To both buffers NaCl was added, so that the total Na^+ concentration was 150 mM. Standard equilibrium exposure time for the C18 fibers was 24 hours. Vials were placed on a Stuart Roller Mixer SRT9D at 60 rpm during exposure. Desorption of the C18 fibers occurred in autosampler vials with insert using 180 µL 90% acetonitrile with 10% Milli-Q water for at least 30 minutes, according to the instructions of the manufacturer.

Overview of sorption experiments

First sorption experiments were based on the comparison of the 7 and 35 µm PA fibers, C18 and the prototype C18/SCX coating. The time needed to equilibrate SPME fibers in amphetamine solutions of 7.4 µM was tested in 150 mM NaCl at pH 7.4, buffered by 10 mM phosphate buffer. Using three separate vials for each time point, PA fibers were exposed in duplicate or triplicate in each vial, whereas C18 and C18/SCX fibers were exposed individually. SPME sorption isotherms in the same physiologically relevant medium were created for all four SPME types and for a range of amphetamine concentrations, using exposure times that guaranteed equilibrium for each fiber.

The influence of pH on sorption of amphetamine to the fibers was determined using different exposure buffers, which ranged in pH between 2 and 12, with approximately 1 pH value increments. All buffers prepared were based on the Na^+ salts of the buffer components. 10 mM acetate buffers were used between pH 4 and 6, and 10 mM carbonate buffers between pH 8 and 10. All other pH solutions consisted of phosphate buffers. The exact pH of all buffers was determined using a pH meter (Knick pH-meter 761 Calimatic). For all buffers, NaCl was added to reach a total Na^+ concentration of 150 mM. Amphetamine from a stock solution in methanol was spiked to the buffers. C18 was tested at two pH values (pH 7.4 and pH 9.0), as the manufacturer discouraged use of the fiber for long time intervals above physiological pH. Exposure time was 24 hours for the PA and C18 fibers and 4 hours for the C18/SCX fiber.

As competition between electrolyte cations and amphetamine for the SCX sites on the C18/SCX fiber was expected, the influence of the medium composition on the equilibrated sorption isotherms was studied. We first compared Milli-Q ($6 < pH < 7$) and PBS (pH 7.4, 146 mM Na^+ and 4.2 mM

K^+). Additional exposure solutions with monovalent cations were 10 mM phosphate buffer (equal composition as the buffer in PBS, described above), 10 mM phosphate buffer with 140 mM Na^+ and 10mM phosphate buffer with 140 mM K^+. Ca^{2+} was used to determine the influence of divalent electrolytes on sorption. To prevent the formation of a precipitate, no buffer system was used for these experiments. Two Ca^{2+} concentrations were tested, a physiological concentration of 2.5 mM (pH 5.9) and a concentration of 50 mM (pH 6.4) as to achieve a solution with a similar ionic strength to 150 mM Na^+. Exposure time was 2 hours. Sorption isotherms were fitted with a Langmuir equation:

$$C_f = \frac{C_{f,max} \cdot b \cdot C_{aq}}{1 + b \cdot C_{aq}} \quad (1)$$

Here, the C18/SCX fiber concentration, C_f, is related to the water concentration, C_{aq}, through a Langmuir constant b • $C_{f,max}$ is the maximum sorption capacity of the fiber for amphetamine. The fiber-water sorption coefficient (D_{fw}) at concentrations well below $C_{f,max}$, in the linear isotherm range, is calculated as b • $C_{f,max}$. The argument for selecting this model is that sorption of cationic compounds to the C18/SCX coating is based on electrostatic interaction with a saturation level at the cationic exchange capacity (CEC) of the coating.
The Langmuir model can also account for sorption competition:

$$C_f = \frac{C_{f,max} \cdot b1 \cdot C_{aq,1}}{1 + b1 \cdot C_{aq1} + b2 \cdot C_{aq2}} \quad (2)$$

where $C_{aq,2}$ is the inhibitory concentration of another compound present in the solution and b_2 is the Langmuir constant of that compound.
When comparing isotherms at the fiber concentrations only in the range readily below the $C_{f,max}$, the estimates of the parameters in the Langmuir model become unreliable. Therefore, isotherms are plotted using a simple Freundlich model:

$$C_f = K_F \cdot C_{aq}^{\,nF} \quad (3)$$

Here, the partitioning of a compound from the aqueous phase to the fiber is modeled by a Freundlich constant K_F and an exponent n_F. As any experimental set-up only defines sorption in specific circumstances, sorption from the aqueous phase to the fiber is expressed by the distribution coefficient (D_{fw}). On a double logarithmic scale, n_F is represented as the slope of the regression line and indicates the linearity of sorption.

LC analysis

The LC system used was a Prominence HPLC, consisting of two pumps, an autosampler, a column oven (set at 40°C), a UV-detector and fluorescence detector, all from Shimadzu ('s-Hertogenbosch, The Netherlands), and a C18 column (GraceSmart RP C18, ID 150 x 2.1 mm, 5 µm particle size, Grace, Breda, The Netherlands). Mobile phase used was a 10 mM phosphate buffer at pH 3.0 with 4.8% (v/v) acetonitrile. For the C18/SCX and C18 fibers, flow rate was 0.4 ml/min and amphetamine was detected using UV at 208 nm. For PA fibers, flow rate was 0.2 ml/min and detection of amphetamine occurred through fluorescence (λ_{ex} 204 nm, λ_{em} 280 nm) as this provided a slightly more sensitive detection of amphetamine at the expected low concentrations of amphetamine. Limit of quantification (LOQ) was set at a signal to noise ratio of 10:1. LOQ using fluorescence is 150 nM while the LOQ using UV detection is 350 nM. Repeated injection of an 18.5 µM amphetamine concentration resulted in a relative standard deviation of 0.53% over 10 consecutive data points.

In the sorption experiments, both the concentrations in fiber as well as aqueous concentrations were always measured. A calibration curve made from aqueous concentrations before exposure was used to calculate the remaining aqueous concentration after exposure. Concentrations in fibers were calculated based on a calibration curve in acidified desorption fluid. Fiber concentrations were then normalized based on the coating volume of the different fibers (349 nL/cm for C18/SCX, 27 nL/cm for PA 7 µm, 154 nL/cm for PA 35 µm and 346 nL/cm for C18). Calculated aqueous and fiber concentrations were used to calculate depletion of aqueous concentrations and to check mass balances to account for all amphetamine added. Repeating a sorption experiment with different fibers yielded no significantly different results, as shown in the Supporting Information, Figure S2.

Data analysis

Data was plotted and analyzed using Graph Pad Prism 6 for Windows. Nonlinear regression of the sorption isotherms was corrected with weighting $1/Y^2$.

RESULTS AND DISCUSSION

Uptake kinetics of amphetamine to PA and C18/SCX fibers

Figure S3 in the Supporting Information shows the uptake kinetics for the different fibers at pH 7.4. Equilibrium was reached in approximately 16 hours for 35 µm PA, 4.5 hours for 7 µm PA and 1.5 hours for C18/SCX. At equilibrium, the D_{fw} of 7.4 µM amphetamine is 0.46±0.01 for 35 µm PA, 5.1±0.2 for 7 µm PA and 205±9 for C18/SCX. As the pKa of amphetamine is 9.9, 99.7% is charged at pH 7.4. This explains the relatively low affinity for the PA coatings, as their sorption mechanism is mostly based on the neutral compound. Sorption of amphetamine to the C18/SCX coating is two orders of magnitude higher, and also much faster than the PA coatings. Whereas neutral amphetamine partitions into the bulky PA polymer, the C18/SCX coating has a more porous structure which leads to a faster uptake as dissolved amphetamine can penetrate the coating before sorption occurs.

Uptake kinetics of amphetamine to the C18/SCX fiber was also fast at pH 11.4 where amphetamine is mainly present in the non-ionized form (about 97%). Exposure to such high pH was shown not to cause coating degradation, as depicted in the Supporting Information, figure S4. For the C18/SCX, equilibrium was reached in 1.5 hours with a D_{fw} of 257±11. Comparing the C18/SCX D_{fw} at pH 7.4 and pH 11.4 shows that the sorption affinity of the cationic species is, presumably by binding to the SCX sites, comparable to the affinity of the neutral compound for the C18-regions of the coating. This shows the beneficial effect of the C18 phase of the fiber, as for other organic sorbent material with high cation-exchange capacities but without this hydrophobic phase, such as soil organic matter (*Droge & Goss 2012*), humic acids (*Karthikeyan & Chorover 2002, Sibley & Pedersen 2008*) and phosphatidylcholine liposomes (*Hunziker et al. 2001*), sorption of the cationic species is higher than of the neutral compound.

Sorption isotherms for PA, C18 and C18/SCX fibers

Because the D_{fw} determined from the uptake experiment relates only to a single exposure concentration, sorption isotherms were made for all three fibers at equilibrium exposure times (24 hours for the PA and C18 fibers, 2 hours for C18/SCX) in the same pH 7.4 buffer with 150 mM Na^+. The data were fitted with a simple Freundlich isotherm (eq. 3) to test for nonlinearity (figure 1).

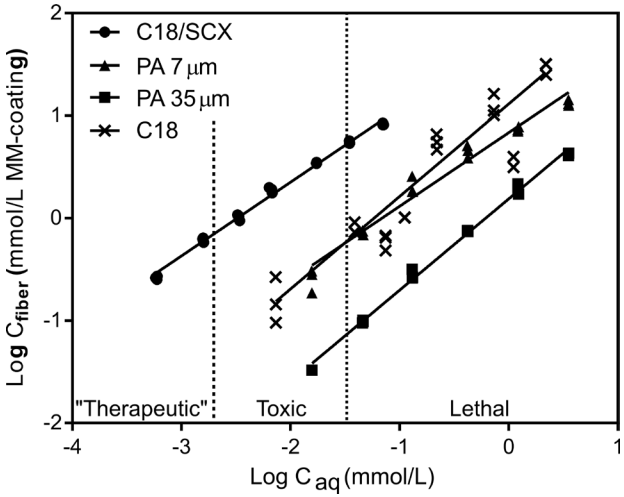

Figure 1. Sorption isotherms for PA fibers with different coating thickness and C18/SCX fibers. Exposure was at equilibrium for all fibers (24 hours for PA and C18, 2 hours for C18/SCX) in a solution composed of 10 mM phosphate buffer at pH 7.4 with 140 mM added NaCl. The affinity of the C18/SCX fibers for amphetamine at pH 7.4 is higher, resulting in an improved sensitivity. As the 7 µm PA fiber contains some cation exchange sites, it performs slightly better than the 35 µm PA fiber. Sorption to the C18 fiber is comparable to sorption to the 7 µm PA fiber.

Sorption of charged amphetamine to the C18/SCX coating is much more favorable than to the C18 or PA coatings over the entire concentration range tested. The C18/SCX fiber is therefore much more sensitive than the PA coatings at physiological pH. Freundlich exponents are 0.89±0.02 for 35 µm PA, 0.72±0.03 for 7 µm PA, 0.90±0.06 for C18 and 0.72±0.01 for C18/SCX, respectively. For the C18/SCX fiber, sorption is expected to be dominated by the protonated amphetamine species, as it is the most abundant at pH 7.4, and thus a process that occurs through electrostatic interactions. Nonlinear isotherms are not uncommon when sorption occurs through electrostatic interactions, as shown for the binding of cationic compounds to humic acids (*Sibley & Pedersen 2008*) and soil organic matter (*Droge & Goss 2012*). As the 7 µm PA fiber has recently been shown to possess a relatively small density of cation exchange sites (*Chen et al. 2012*), it is not surprising that sorption coefficients are higher compared to 35 µm. However, as the slope of the 35 µm fiber is also slightly lower than 1, we suspect this PA coating to also have

some cation exchange capacity, although very little in comparison to the 7 µm PA fiber.

For the C18 fiber, we also observe some nonlinearity in the sorption isotherm. We believe this may be the result of charged silanol groups in the coating. The pKa of these silanol groups is 6.8 (*Escher et al. 2000*), and sorption of cationic amphetamine (at pH 7.4) to these groups can contribute to the total sorption. We believe deprotonation of silanol groups also occurs for C18/SCX but as this coating already possesses strong cation exchange groups, the contribution of the free silanol groups to the sorption to the C18/SCX fiber is much smaller. This could also explain the large variation between the different data points of the C18 fiber, as the sorption is dependent on the availability of free silanol groups.

When examining the 7 µm PA data in more detail, it appears that sorption is first linear and levels off at higher aqueous concentrations (see Supporting Information, Figure S5). Splitting these data results in a slope of 0.98±0.07 at low concentrations and 0.51±0.04 for the higher concentrations. Although the number of data points are limited, linearity seems to continue up to around 3 mmol/L PA coating, comparable to the concentration of 2 mmol/L found by (*Chen et al. 2012*) for the sorption of quaternary ammonium compounds to the same 7 µm PA fibers. Since the cation exchange capacity was found to be 30 mmol/L coating for the 7 µm PA fiber, the nonlinear isotherm region most likely indicates the transition phase from sorption dominated by ion-exchange sites towards sorption dominated by bulk partitioning of the neutral species, corresponding to an affinity close to the 35 µm PA as this has the least cation exchange sites (*Chen et al. 2012*). Our highest tested concentrations are already above reported lethal concentrations of amphetamine in humans (*Baselt 2004*) (see figure 1), so studying concentrations in this region in more detail was beyond the scope of this study.

Effect of pH on amphetamine sorption to PA, C18 and C18/SCX fibers

Figure 2A shows sorption to the three coatings at different pH. All buffers had equal ionic strength although different buffer systems were used. However, this is not a confounding factor, as the different buffer systems used result in comparable sorption at equal pH (see Supporting Information, Figure S6). The consistent increase of D_{fw} with higher pH shows once more that the PA and C18 fibers mainly sorb the neutral fraction of amphetamine, which results in the highest observed D_{fw} values above the pKa of amphetamine.

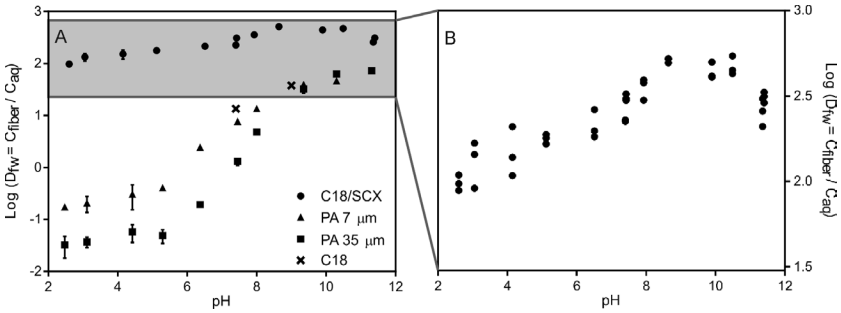

Figure 2. pH dependence of SPME fibers. Exposure solution was a 10 mM buffer with addition of 140 mM NaCl. Phosphate buffers were used with exception of data between pH 4 and 6 (acetate) and pH 8 and 10 (carbonate). (A) Comparison of C18/SCX with conventional fibers (C18, 7 µm and 35 µm PA) shows that C18/SCX sorbs both cationic and neutral amphetamine while the conventional fibers mainly sorb the neutral species. Data was measured in triplicate but depicted as mean ± SEM. C18/SCX data includes a second triplicate at pH 7.4 and pH 11.4. (B) Triplicate measurements (except for pH 7.4 and pH 11.4 where n=6) for C18/SCX fiber show a slight increase in D_{fw} at pH ranging between 2 and 9 due to a decrease in the presence of H^+ as a competitor. Sorption of amphetamine at pH 7.4 (99.7% cationic) and pH 11.4 (97% neutral) is comparable.

As the neutral fraction is the dominating sorptive species for the 35 µm PA fiber, the pKa of amphetamine can be deduced from the experimental data (see Supporting Information, Figure S7). Calculated pKa of amphetamine is 9.5, which is in good accordance with the reported pKa in literature of 9.9 (*Baselt 2004*). As we suspect the 35 µm PA fibers to also have a small cation exchange capacity, the actual pKa may be even closer to the reported pKa. As the pKa of the carboxylic groups in PA is estimated at 4.7 (*ChemAxon, www.chemicalize.org*), these sites are unavailable for sorption of cationic amphetamine at low pH. This explains why D_{fw} starts to increase from around pH 6, as then many carboxylic groups will be charged and sorb cationic amphetamine, which is the only species present at that pH. The overall higher affinity for 7 µm PA compared to the 35 µm PA at pH<pKa indicates the presence of more cation exchange sites.

Having a much higher cation-exchange capacity than the 7 µm PA, the C18/SCX coating shows nearly constant log D_{fw} (range 1.9-2.7) at every pH tested, demonstrating comparable sorption affinity of both cationic and neutral amphetamine and therefore the wide functionality of this prototype SPME fiber coating.

Figure 2B shows the data for the C18/SCX coating in more detail. Between pH 2.6 and pH 8.6, sorption coefficients increase with increasing pH (0.8 log units). This may be related to the reduced competition effects by decreasing H^+ concentrations with higher pH values, although this concentration becomes very small at high pH. Here, sorption may be increased due to the deprotonation of the silanol groups in the fiber coating, as these have a reported pKa of 6.8 (*Escher et al. 2000*). Protonation of propylsulfonic acid should not occur for the C18/SCX fiber, as the pKa is <1, which means that the propylsulfonic acid is completely ionized over the entire pH range. Still, the difference between sorption coefficients at for instance pH 4 and pH 7 is merely half a log unit, much less than observed in the PA coatings.

Cation exchange capacity of the C18/SCX fiber coating

As shown in figure 3, sorption of high concentrations of amphetamine in Milli-Q water results in saturation of the C18/SCX fiber. Therefore, we chose to apply the Langmuir model to fit the sorption data (eq. 1). The Langmuir model resulted in the following parameters: $C_{f,max}$ is 47±2 mmol/L and b_1 is 498±36. In the Langmuir model, sorption appears linear at concentrations in the fiber below 10% of $C_{f,max}$. This apparent $C_{f,max}$ is only 1.5 times higher than the measured CEC for 7 µm PA, and does not reflect the 20x stronger sorption of amphetamine to the C18/SCX at e.g. pH 7.4 (figure 2). A higher CEC was also expected based on the density of S in the coating. We have not tested sorption of amphetamine at concentrations above 1.9 mM so sorption may still increase nonlinearly to higher fiber concentrations at higher dissolved amphetamine concentrations.

The sorption isotherm of amphetamine in PBS also seems to level off to a saturation capacity, albeit at higher C_{aq} compared to exposure in Milli-Q. The shift in the sorption isotherm between Milli-Q and PBS may simply relate to competition effects with other cations present in the buffer solution (Na^+, K^+). The experimental data were fitted with the competition model (eq. 2). We used the same b_1 value for amphetamine that was estimated from the data in Milli-Q water, since this may be considered as an intrinsic property of

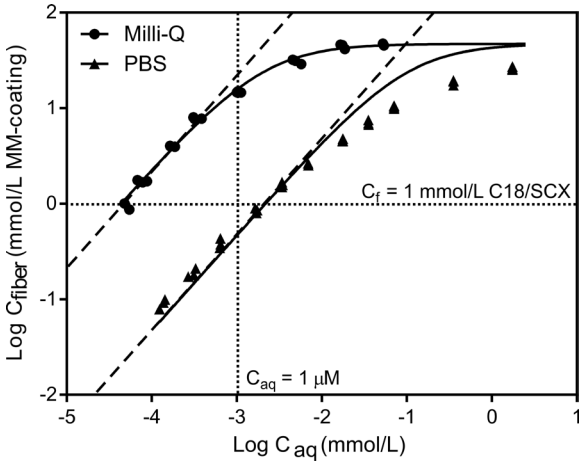

Figure 3. Graphical display of Langmuir equation as sorption model. Exposure solutions used were Milli-Q water (6<pH<7 to ensure all amphetamine is charged) and PBS (pH=7.4). Curve for PBS was simulated using $C_{f,max}$ and b1 of amphetamine as determined from the curve for Milli-Q water. $C_{aq,2}$ was set at 150 mM. Dashed lines indicate linearity, dotted lines represent concentrations used for D_{fw} calculations in Table 1.

amphetamine in the near absence of competitive cations in Milli-Q. We also applied the same $C_{f,max}$ for PBS as for Milli-Q water, as there is no reason to assume the competition is irreversible and would lead to a lower CEC. Assuming Na^+ as the main electrolyte cation, concentration $C_{aq,2}$ in PBS is equal to 150 mM, and b_2 was calculated to be 0.33±0.03, which suggests a sorption coefficient ($C_{f,max} \cdot b_2$) of Na^+ of 16 (L_{aq}/L_{fiber}). The Langmuir curve of eq. 2, however, does not fit to the whole data range. Again, at very low concentrations, sorption to the C18/SCX fiber is linear, but sorption starts to level off already at concentrations below 10% of $C_{f,max}$. The sorption of charged amphetamine to ion-exchange sites in the presence of other cations may be a more complex sorption process than the Langmuir competition model can account for. A more refined modeling approach should probably incorporate electrostatic attraction as well, instead of assuming competition only. Apparently, the influence of specific medium constituents complicates the use of C18/SCX fibers. Calibration of the SPME method will be required for each appropriate test medium as long as the underlying mechanisms cannot be modeled adequately.

Competition effects on C18/SCX fiber coating

The competition effects of Na^+, K^+ and Ca^{2+} on the sorption of amphetamine to the C18/SCX fiber were tested. Table 1 shows the fitted parameters, which represent the sorption coefficient D_{fw} at 1 µmol/L and the Freundlich exponent (n). As sorption is decreased at higher aqueous concentrations when the fiber concentrations approximate the CEC, the difference between log D_{fw} values at various aqueous concentrations does not remain equal over the entire concentrations range. The calculated D_{fw} values at a single fiber concentration are therefore more comparable as this relates to the same bound fraction of the CEC. D_{fw} values were calculated at a fiber concentration of 1 mmol/L coating (see table 1), as figure 3 shows that sorption at this concentration is expected to be linear.

Figure 4 shows the influence of different medium compositions on the sorption of amphetamine to the C18/SCX fiber. Since the tested concentration ranges did not include sufficient data near the ion-exchange capacity, data were fitted with the Freundlich isotherm (eq. 3). The sorption isotherm in a 10 mM phosphate buffer (8 mM Na^+, 2 mM K^+, pH 7.4) serves as a reference. When adding 140 mM of Na^+ to this buffer, the sorption affinity is significantly decreased (0.6 log units at C_f = 1 mmol/L coating). The addition of 140 mM of K^+ to the buffer results in an even stronger decrease in sorption affinity compared to the buffer (0.9 log units at C_f = 1 mmol/L coating). The difference between equal concentrations of K^+ and Na^+ on C18/SCX sorption affinity is 0.3 log units (at C_f = 1 mmol/L coating). Interestingly, similar effects at equal ionic strength solutions with K^+ and Na^+ were observed for the binding of clarithromycin to humic acid (*Sibley & Pedersen 2008*), and metformin to sulfonated cation exchange polymer (*Bäuerlein et al. 2012*). Here, competitive effects of monovalent electrolytes on sorption were also studied and the effect of K^+ was slightly larger than for Na^+. K^+ may have a stronger association with the negatively charged sulfonic groups because it has a smaller hydrated radius and can therefore interact at a smaller distance (*Volkov et al. 1997*). This causes K^+ to be more difficult to displace and therefore, it may have a larger competitive effect (*Murakami 1980, Sibley & Pedersen 2008*).

The influence of divalent electrolytes was tested using Ca^{2+} in two concentrations, 2.5 mM and 50 mM. These concentrations have an approximate equal ionic strength to the Na^+ concentrations in the 10 mM buffer reference and 140 mM Na^+, respectively. Comparison of the sorption

	Log D_{fw} ± SE (C_{aq} = 1 µM)	Log D_{fw} ± SE (C_f = 1 mmol/L C18/SCX)	n_F ± SE	R^2
Milli-Q water[a]	4.19 ± 0.03	4.36 ± 0.12	—	0.98
PBS[a]	2.67 ± 0.07	2.66 ± 0.07	—	0.75
Buffer (8 mM Na^+ & 2 mM K^+)	3.07 ± 0.03	3.10 ± 0.05	0.66 ± 0.01	0.98
Buffer with 140 mM Na^+	2.63 ± 0.03	2.49 ± 0.03	0.72 ± 0.01	0.99
Buffer with 140 mM K^+	2.39 ± 0.03	2.21 ± 0.03	0.78 ± 0.01	0.99
2.5 mM Ca^{2+}, no buffer	2.92 ± 0.04	2.89 ± 0.05	0.71 ± 0.02	0.97
50 mM Ca^{2+}, no buffer	2.73 ± 0.04	2.60 ± 0.05	0.66 ± 0.02	0.98

Table 1. Variables D_{fw} and n_F for sorption from solutions of different ionic composition to C18/SCX fiber. For Milli-Q and PBS, data is from figure 3; for other matrices, data is from figure 4.

[a] Derived from Langmuir isotherm, so n_F is not indicated.

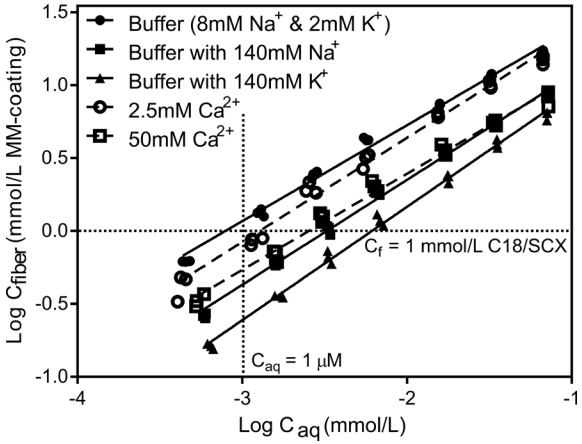

Figure 4. Sorption isotherms for several exposure solutions with different electrolytes. Exposure solutions include a 10 mM phosphate buffer alone (closed circles), with 140 mM Na^+ (closed squares), with 140 mM K^+ (closed triangles), Milli-Q water with 2.5 mM Ca^{2+} (open circles and dashed line) and with 50 mM Ca^{2+} (open squares and dashed line). pH of all phosphate buffers was 7.4, pH of the Ca^{2+} solutions was 5.9 at 2.5 mM and 6.4 at 50 mM. Exposure time for all isotherms was 2 hours. Dotted lines represent concentrations used for D_{fw} determination in Table 1.

isotherms shows that 2.5 mM Ca^{2+} and buffer have a similar influence on the sorption of amphetamine (0.2 log units at C_f = 1 mmol/L coating), and also that 50 mM Ca^{2+} does not result in a stronger competition than 140 mM Na^+ (0.1 log units at C_f = 1 mmol/L coating). This strongly contradicts with findings for organic matter in Na^+ and Ca^{2+} solutions, where sorption was reduced 1 log units more in the presence of Ca^{2+} (with equal IS) compared to the Na^+ competition (*Chen et al. 2012, Droge & Goss 2012*). We expect this to be the result of preferential sorption of Ca^{2+} to carboxylic groups (majority of sites at neutral pH in humic acid) compared to sulfonic groups (present in C18/SCX). This would result in an enhanced competitive effect for Ca^{2+} on carboxylic ion-exchange sites, as was also shown for enhanced cationic drug release from sulfonic polymer fibers compared to carboxylic polymer fibers (*Jaskari et al. 2001*). A recent sorption study on different solid phase extraction materials, however, showed a similar enhanced competition of Ca^{2+} for both carboxylated and sulfonated cation-exchange polymer (*Bäuerlein et al. 2012*).

The dominant sorption mechanism of amphetamine to the C18/SCX coating at pH 7.4 is based on ion-exchange. The presence of other cations in the solution results in lower sorption of amphetamine due to competition. At equal ionic strength, competition follows the order Ca^{2+}=Na^+<K^+. For the practical application of this fiber coating, it is important to take these competition effects into account, for example by carefully selecting the medium when calibrating the fiber measurements.

CONCLUSIONS

Our current study aims at getting a mechanistic insight into the sorption of amphetamine to different SPME coatings, in the end resulting in SPME methods that will be applicable in *in vivo* and forensic studies. We have successfully shown that the prototype "mixed-mode" C18/SCX fiber has several advantages over the conventional fibers with PA or C18 coating.

The affinity of amphetamine at pH 7.4 for the C18/SCX fiber is 20 times greater than for the 7 µm PA fiber and 180 times greater than for the 35 µm PA fiber. The C18/SCX fiber can therefore be used to measure more sensitively in the µM range, which includes therapeutic concentrations for many drugs, which is infeasible for the PA coatings and most likely also other conventional coatings. However, at higher concentrations, saturation of the sorption sites of the C18/SCX fiber may occur and sorption becomes less well predicted.

Additionally, several factors such as other cations and pH have competitive effects on sorption to the fiber, which requires careful calibration of the fiber in an appropriate matrix.

The sorption data for the C18/SCX fiber clearly show that both the ionic and the neutral form of amphetamine have affinity for the fiber coating. Cationic sorption is a saturation process, reaching a maximum around 47 mmol/L C18/SCX coating. It would be logical to assume this cation exchange capacity to be equal for all cationic drugs. For amphetamine, the ion-exchange affinity of the charged species for the C18/SCX fiber is comparable to the affinity of neutral amphetamine for the same fiber. We expect that organic cations with more hydrophobic structures sorb stronger to the C18/SCX coating, and that more polar cations may display lower sorption coefficients. At much higher affinities, test systems may be readily depleted upon introduction of the 15 mm fiber, and competition in mixtures may be another issue of concern. It seems that lower affinities of more polar organic cations, as is demonstrated here with amphetamine, would still render the C18/SCX useful for sensitive analysis of freely dissolved concentrations at pH<<pKa.

Future work will include expanding the data set with sorption data for other pharmaceutical and illicit cationic drugs to the C18/SCX or "mixed-mode" fiber and applying the fiber in biological fluids and *in vivo*.

Acknowledgement

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SUPPLEMENTARY DATA

Desorption of C18/SCX fiber

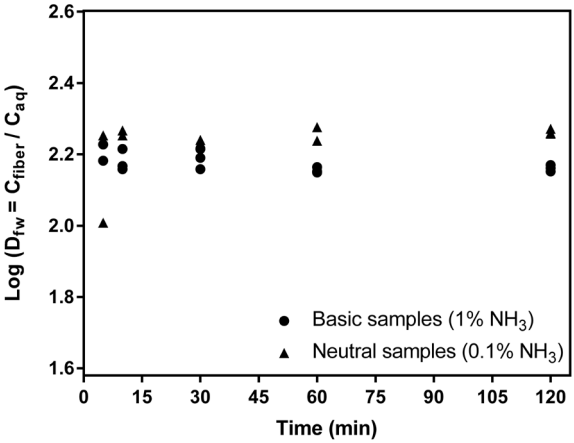

Figure S1. Desorption of mixed-mode fibers. Fiber concentrations were determined after an extraction of 120 minutes of a 74 µM AMP concentration in PBS with 0.9 mM $CaCl_2$. For the basic samples, desorption was 120 µL 90% acetonitrile and 10% Milli-Q with 10% NH_3 in the water phase (total NH_3 concentration 1%) acidified with 60 µL 0.1 M HCl, reaching an end pH of 9.4. For the neutral samples, this was 120 µL 90% acetonitrile and 10% Milli-Q with 1% NH_3 in the water phase (total NH_3 concentration 0.1%) acidified with 60 µL 0.1 M HCl, reaching an end pH of 2.2. For both desorption fluids, complete desorption occurs within 10 minutes. Assumed was that a 15 minute desorption would be sufficient to completely desorb the fiber. Carry-over was determined for all fibers. For the fibers in desorption fluid with 1% NH_3, carry-over was between 0% and 1.5%. For 0.1% NH_3, this was between 1% and 3%. In the current manuscript, we chose to use desorption fluid with 1% NH_3 and injected samples with relatively high pH. However, this proved to have a negative effect on the lifetime of our HPLC column.

Reproducibility

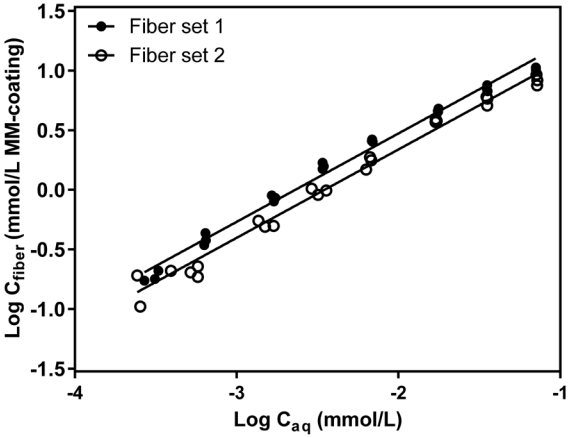

Figure S2. Reproducibility between experiments performed with different fibers. Sorption isotherms of AMP in PBS with 0.9 mM $CaCl_2$ (pH 7.4) at equilibrium exposure time (120 minutes). Both sets of fibers had been used for other experiments prior to this experiment. There is no significant difference in D_{fw} or slope (p=0.13 and 0.72, respectively) as determined by the extra sum-of-squares F test.

Extraction time profiles for different fibers

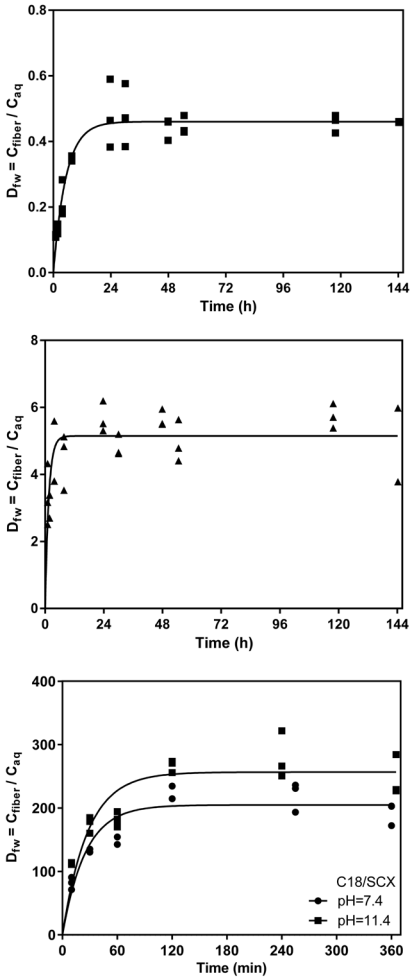

Figure S3. Extraction time profiles for (A) 35 µm PA, (B) 7 µm PA and (C) C18/SCX. Please note the difference in X- and Y-axis between the different graphs. All fibers were exposed in a 10 mM phosphate buffer at pH 7.4 with NaCl added to a total ionic strength of 150 mM. For the new C18/SCX coating, an extraction time profile was also made at pH 11.4 to investigate sorption of the neutral compound. Exposure solution here was also a 10 mM phosphate buffer with added NaCl to a total ionic strength of 150 mM.

C18/SCX coating degradation at pH 11.4

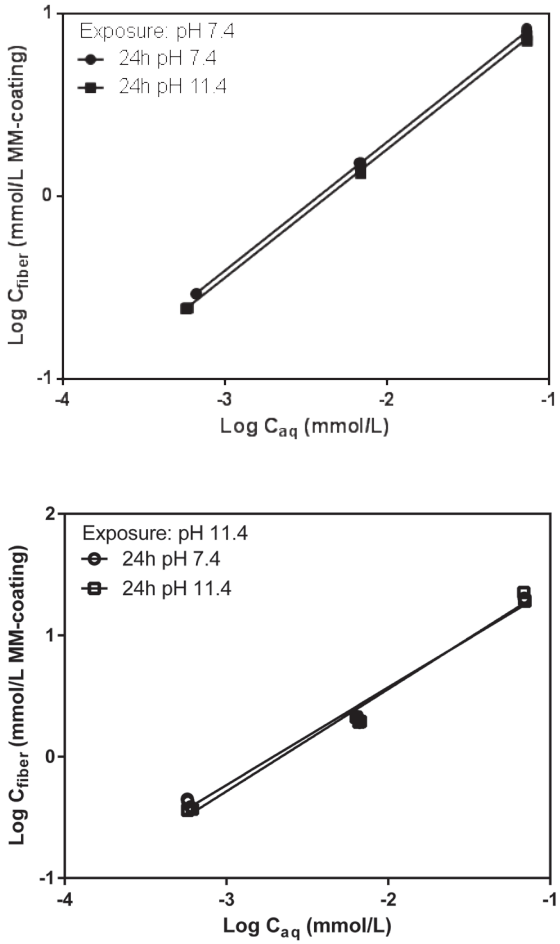

Figure S4. Results of coating degradation experiment with C18/SCX fibers. Fibers were exposed to buffers of pH 7.4 or pH 11.4 for 24 hours. After this, the fibers were exposed to amphetamine concentrations in a 10 mM phosphate buffer at (A) pH 7.4 and (B) pH 11.4. For sorption at both pH, neither D_{fw} nor slope are significantly different (pH 7.4; p=0.07 and 0.92, pH 11.4; p=0.51 and 0.33, respectively) as determined by the extra sum-of-squares F test.

Sorption isotherm for 7 µm PA fiber

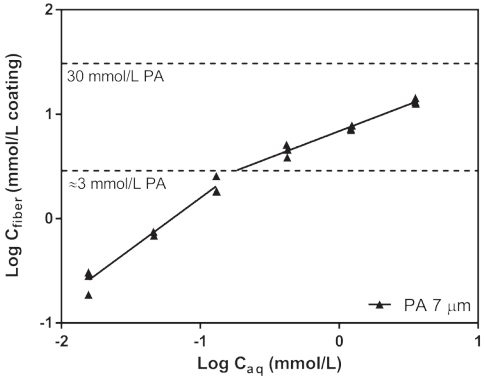

Figure S5. Data from the sorption isotherm of 7 µm PA split into two parts. The first part shows linearity of uptake up to a fiber concentration of around 3 mmol/L PA coating (slope is 0.98). In the second part, data points are non-linear (slope is 0.51). Dotted lines represent fiber concentrations of 3 mmol/L as the cut-off value for linearity and 30 mmol/L as the CEC found by Chen et al.[1]. As the highest tested amphetamine concentration here are already above reported lethal concentrations in humans, testing even higher concentrations was beyond the scope of this study.

1. Chen, Y.; Droge, S. T. J.; Hermens J. L. M. *J.Chromatogr.A.* **2012**, *1252*, 15-22.

Influence of buffer system on sorption

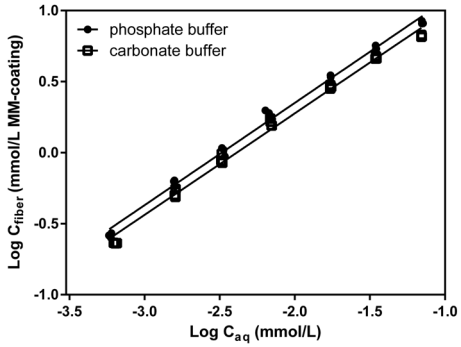

Figure S6. Influence of type of buffer choice on AMP partitioning. Sorption isotherms for a 120 minute extraction of AMP from two different buffer systems. Buffer used was either a 10mM phosphate buffer at pH 7.4 or a 10mM carbonate buffer at pH 7.4. NaCl was added to both buffers to a total ionic strength of 150mM. Both D_{fw} and slope are not significantly different (p=0.09 and 0.82, respectively) as determined by the extra sum-of-squares F test.

pH dependent sorption for 35 µm PA fiber

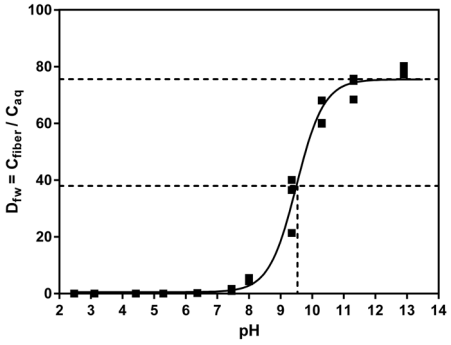

Figure S7. Sorption coefficients for 35 µm PA fiber at different pH. By plotting on a normal Y-axis, a sigmoidal curve can be plotted and the pKa of amphetamine can be determined as this is the pH at which 50% of the maximum D_{fw} is reached. Here, pKa of amphetamine is 9.5 which is in good accordance with the reported pKa in literature of 9.9. If the 35 µm PA fiber possesses some cation exchange sites, the estimated value is slightly lower than the actual pKa.

SORPTION OF AMITRIPTYLINE AND AMPHETAMINE TO MIXED-MODE SPME IN DIFFERENT TEST CONDITIONS

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ABSTRACT

A solid-phase microextraction (SPME) method based on a sampler coating that includes strong cation groups (C18/SCX) is explored as a rapid direct sampling tool to detect and quantify freely dissolved basic drugs. Sampling kinetics, sorption isotherms and competitive effects on extraction yields in mixtures were tested for amphetamine and the relatively large/hydrophobic tricyclic antidepressant amitriptyline. Both compounds are >99% ionized at pH 7.4 but their affinity for the C18/SCX fiber is markedly different with distribution coefficients (D_{fw} values) of 2.49±0.02 for amphetamine and 4.72±0.10 for amitriptyline. Typical changes in electrolyte homeostasis that may occur in biomedical samples were simulated by altering pH and ionic composition (Na^+ and K^+ concentrations). These changes were shown to affect C18/SCX sorption affinities of the tested drugs with less than 0.2 log units. At relatively low fiber loadings (<10 mmol/L coating) and at all tested exposure times, linear sorption isotherms were obtained for both compounds but at aqueous concentrations of the individual drugs corresponding to concentrations in blood that are lethal, sorption isotherms became strongly nonlinear. Competition effects within binary mixtures occurred only if combinations of aqueous concentrations resulted in total fiber loadings that were in the nonlinear range of the SPME sorption isotherm for the individual compounds. We also compared sorption to the (prototype) C18/SCX SPME coating with analogue (biocompatible) C18 coated SPME fibers. C18/SCX fibers show increased sorption affinity for cationic compounds compared to C18 fibers, as tested using amitriptyline, amphetamine and trimethoprim. Surprisingly, sorption affinity of these ionized compounds for the C18 SPME fibers were within 1 log unit of the C18/SCX SPME fibers. This shows that the strong cation exchange groups within the C18/SCX coating only has a relatively small contribution to the total sorption affinity of cationic compounds. Also the role of negatively charged silanol groups in both the C18 and C18/SCX coating seems small, as anionic diclofenac species sorbed strongly to the C18 fiber. Ionized organic species seem to be substantially adsorbed to the high surface area of C18 in SPME types using porous silica based coatings.

INTRODUCTION

Solid-phase microextraction (SPME) was developed in the early 1990s by Arthur & Pawliszyn (*Arthur & Pawliszyn 1990*), and is a simple partition-based extraction technique with several advantages over the conventional sampling methods. New developments and applications of SPME are published on a regular basis, for instance on newly available coatings (*Xu et al. 2013*) and on automation of the sampling method for high-throughput analysis (*Vuckovic et al. 2010*). SPME sampling has unique characteristics, such as the yield of clean concentrated extracts from heterogeneous matrices (*Furton et al. 2000, Kabir et al. 2013*) and negligible depletion of the system that allows for repeated sampling without disturbance of that system (*Heringa & Hermens 2003, Vaes et al. 1996*). At present, SPME has been validated for many different bioanalytical and forensic studies, including *ex vivo* and *in vivo* sampling for a variety of pharmaceuticals (*Kabir et al. 2013, Kataoka & Saito 2011, Lord et al. 2003, Musteata et al. 2008, Ouyang et al. 2011, Prada & Furton 2012, Schubert et al. 2007, Togunde et al. 2012, Vuckovic et al. 2011b, Wang et al. 2011, Zhang et al. 2012, Zhou et al. 2008*). Of particular interest are minimally invasive studies into the biokinetics of pharmaceuticals and drugs. The challenge here is that the majority of drugs of interest in screening procedures are polar ionizable chemicals, of which most exist largely as ionic species at physiological conditions, while conventional SPME sampling has largely focused on extracting neutral compounds, or neutral fractions of ionizable compounds, and may have an insufficient yield for ionized polar drugs.

We have previously described the sampling process of the basic drug amphetamine (pKa 9.9 (*ChemAxon, www.chemicalize.org*)) from physiological medium using a newly developed "mixed-mode" coating (C18/SCX), consisting of hydrophobic C18 chains and embedded strong cation exchange groups (propylsulfonic acid) coated on porous silica (*Peltenburg et al. 2013*). This C18/SCX fiber was shown to extract ionized amphetamine with a much higher yield than other, neutral polymer coatings such as polydimethylsiloxane (PDMS) and polyacrylate (PA). The same benefit of this C18/SCX fiber over conventional SPME coatings was recently shown for a cationic surfactant (*Wang et al. 2013*). Also, this surfactant displayed a much higher affinity to the C18/SCX coating than amphetamine. This indicates that the chemical application range of C18/SCX possibly includes small and polar, but also relatively hydrophobic basic compounds, and that the impact of competing ionic drugs for binding to the C18/SCX coating needs to be examined. A comparable custom-made mixed-

mode SPME coating already showed increased metabolite coverage compared to other conventional fibers in an untargeted metabolomics profiling study (*Vuckovic & Pawliszyn 2011*). This mixed-mode fiber was consequently applied in three *in vivo* global metabolomics studies: in the blood of mice (*Vuckovic et al. 2011a*), in liver and lung tissue of pigs (*Bojko et al. 2013*) and in the brain of rats (*Cudjoe et al. 2013*). These studies show that coatings with ion-exchange phases may especially provide high yields for small organic cations, where the neutral fraction has insufficient affinity to sorb to classic SPME coatings. However, the sorption process is dependent on several factors such as pH and the concentration of other (inorganic) cations such as Na^+ and K^+ (*Peltenburg et al. 2013*) which may compete for sorption to the C18/SCX coating. Although these variables have been studied in equilibrated systems, other factors that influence sorption to C18/SCX coatings, such as the influence of uptake kinetics and the effects on sorption affinity in the presence of other charged drugs in mixtures are still not completely understood. The effects of these variables and test conditions need to be further investigated for a proper application of the C18/SCX sampling method for basic drugs in biological samples.

Our previous work was based on equilibrium sampling of amphetamine in simple physiological buffers to compare the C18/SCX fiber to other SPME coatings (*Peltenburg et al. 2013*). Detailed insight into the characteristics and mechanisms of the extraction process is needed before it can be applied in toxicity or forensic studies. Having these insights is relevant for the relatively new C18/SCX coating where more complex interactions of ionized chemicals with the fiber coating may occur. The current study investigates the influence of pH, ionic composition, temperature, exposure time and agitation on the sorption to the C18/SCX fiber, both for amphetamine as well as amitriptyline. These factors need to be carefully characterized as they can influence sorption to the fiber during applications in in vitro and *in vivo*. After death, especially, pH and ionic composition may change and this may influence sorption to the fiber. Postmortem, blood pH drops from 7.4 to around 5.5 due to an accumulation of acidic glucose metabolites (*Donaldson & Lamont 2013*). The ionic composition of blood changes as Na^+ concentration decreases and K^+ concentration increases due to failure of the Na^+/K^+/ATPase pump (*Yarema & Becker 2005*). Furthermore, the C18/SCX fiber was exposed to mixtures of amphetamine and amitriptyline to study potential competition effects on sorption affinities within mixtures. Another topic that is specifically addressed in this study is the comparison of the affinity of ionized drugs to

the mixed mode (C18/SCX) coating to their affinity for analogue C18 coated SPME fibers that lack the SCX functionality. Such a comparison may provide a more refined rationale on which interactions govern extraction efficiencies to mixed-mode SPME coatings. This study leads to general knowledge that can easily be utilized in more applied studies with these two chemicals, but also in studies with other positively ionized compounds.

MATERIALS AND METHOD

Chemicals and materials

Solid-phase microextraction fibers with mixed-mode (C18/propyl-sulfonic acid; C18/SCX) coating were prototype fibers provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). The fibers were 3 cm pieces of nitinol wire (202 µm diameter) with 1.5 cm of coating at an average thickness of 45 µm (total fiber volume 524 nL). Both C18 and propylsulfonic acid are bonded on porous HPLC column grade silica material which is then bound to the wire with a biocompatible polymeric binder (*Supelco, pers.comm.*). Analogous SPME-LC fiber probes (functional group C18) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). These C18 fibers (fiber volume 520 nL) also consist of a 1.5 cm coating at an average thickness of 45 µm, coated on nitinol wire (200 µm diameter). Amphetamine hydrochloride was purchased from Spruyt Hillen, IJsselstein, The Netherlands. Amitriptyline hydrochloride and diclofenac sodium were from Sigma Aldrich. Trimethoprim was from Dr. Ehrenstorfer (Augsburg, Germany). Phosphate buffered saline (PBS) consisted of 138 mM NaCl, 8 mM of Na_2HPO_4, 1.5 mM of KH_2PO_4 and 2.7 mM KCl (all Merck, Darmstadt, Germany) dissolved in Milli-Q water (18.2 MΩ·cm, Millipore, Amsterdam, The Netherlands). Calcium dichloride (Sigma Aldrich) was used to make Dulbecco's PBS (DPBS) (*Dulbecco & Vogt 1954*), i.e. PBS containing 0.9 mM Ca^{2+}. Buffers of different pH were either phosphate buffers (H_3PO_4 and $Na\ H_2PO_4$ between pH 2 and 4, $Na\ H_2PO_4$ and Na_2HPO_4 between pH 6 and 8, and Na_2HPO_4 and Na_3PO_4 above pH 11), acetate buffers (CH_3COOH and CH_3COONa between pH 4 and 6) or carbonate buffers ($NaHCO_3$ and Na_2CO_3 between pH 8 and 11). Borate buffer pH 10 consisted of H_3BO_3 adjusted to pH 10 with NaOH. All salts were from Merck or Sigma Aldrich. Ammonia solution (25%) was obtained from Merck. Methanol and acetonitrile were HPLC-grade (BioSolve, Valkenswaard, The Netherlands).

SPME procedure

Test solutions were spiked from stock solutions in methanol, ensuring methanol fractions of <1%. During SPME fiber exposure, samples were either placed on a roller mixer for agitated sampling (40 rpm) or kept on the lab table during static sampling. These roller mixers (Stuart SRT9) are specially designed to provide a gentle but effective agitation, which is needed for future work with cells or proteins. After a certain exposure time, fibers were transferred to vials containing 120 µL desorption fluid. Fibers are wiped gently to remove any droplets of buffer before placing them in desorption fluid. For C18/SCX fibers, this consisted of 90% acetonitrile and 10% Milli-Q water with 0.1% NH_3 (of end volume) with a pH around 11 to extract the neutral base. The fibers were desorbed for more than 96% within 15 minutes (Supporting Information, figure S1). After taking out the desorbed fibers, the desorption solutions were acidified to pH 2-3 using 60 µL 0.1 M HCl, to approximate the mobile phase (*Peltenburg et al. 2013*). C18 fibers were desorbed for a minimum of 30 minutes in 90% acetonitrile and 10% water (180 µL), according to the instructions of the manufacturer. To re-use the fibers for several experiments, they were pooled after use, kept in desorption fluid overnight and then stored in 50/50 methanol/water until the next experiment. Fiber blanks were incorporated in every experiment in triplicate from exposure solutions that had not been spiked to confirm that no carry-over existed between experiments. Sorption isotherms for amphetamine were shown to be reproducible for fibers from different batches (Supporting Information, figure S2). Sorption isotherms at pH 7.4 of amphetamine comparing the same fibers new and after twenty experiments showed a small decrease of 0.1-0.4 log units in sorption capacity after several uses (Supporting Information, figure S2). Furthermore, new fibers were repeatedly exposed to 3.6 µM amitriptyline to test whether coating degradation was visible after several exposures. This showed no decrease in fiber concentration with repeated exposure (Supporting Information, figure S2), not even when exposed to desorption fluid overnight. Although the fibers are intended for single use, the fibers were regularly checked to ensure no decrease in sorption capacity occurred during repeated use. If this was the case, new fibers were used for the next experiment.

Comparison of C18/SCX and C18 coatings and fiber selection

Sorption of amphetamine and amitriptyline was tested at equilibrium over a wide pH range for both the C18/SCX and C18 fiber to evaluate the role of SCX groups and for quantification of the ion-exchange capacity. Equilibrium exposure times for amphetamine and amitriptyline were calculated from

their uptake curves to the C18/SCX fiber (see Supporting Information, figure S3). To ensure that equilibrium was always reached and from a practical point of view, exposure times used were 2 hours for amphetamine and 3 days for amitriptyline. As amitriptyline has a much higher affinity for the fiber coating than amphetamine, and with that the relative amount of drug transfer to the coating is much higher, this results in a much longer equilibration time for amitriptyline compared to amphetamine.

Equilibration times of amphetamine and amitriptyline to the C18 fiber were assumed to be similar to those for the C18/SCX fiber. For all pH values, 10 mM buffers (all Na-salts) were used with additional 140 mM NaCl. Acetate buffers were used between pH 4 and 6, carbonate buffers were used between pH 8 and 10. Phosphate buffers were used for all other pH values. Amphetamine concentration was 7.4 µM in all samples and amitriptyline concentration was 0.36 µM.

As high pH might damage the fiber coating of the C18 and C18/SCX fiber by damaging the silica particles, measurements of sorption affinity at high pH might not be reliable. For the C18 fiber, the manufacturer recommends that the pH working range is 1-9 but that prolonged exposures >2 hours at pH levels >7.5 could slowly damage the silica. The C18/SCX fiber is produced in the same manner as the C18 fiber. As we are interested in the sorption of the charged as well as the neutral form, we tested the pH dependency of sorption of trimethoprim, a weaker base than amphetamine and amitriptyline (pKa = 7.2 (*ChemAxon, www.chemicalize.org*)), to both fiber types at various pH. As the pKa for this base falls within the working range of the fiber, the measured sorption affinity of the charged and the neutral species should both be obtained without fiber damage. Trimethoprim concentration was 3.4 µM in 10 mL of buffer with fibers exposed for 24 hours.

Furthermore, the silica support material in both C18 and C18/SCX SPME coatings may contain negatively charged silanol groups at pH>5 (*Escher et al. 2000*), which could additionally sorb cationic compounds such as amitriptyline and amphetamine to C18/SCX SPME, and equally to C18 SPME. We hypothesized that this proposed negative charge in C18 SPME should result in strong repulsion of organic anions relative to their neutral species. We therefore also examined the sorption affinity of the acidic drug diclofenac (pKa 4.0 (*ChemAxon, www.chemicalize.org*)) to the C18 fiber at various pH. The diclofenac concentration was always 1.7 µM in 10 mL of buffer and fibers were exposed for 3 days.

Investigating the effects of different test conditions for sampling with C18/SCX fibers

Influence of pH and ionic composition on SPME affinity

The influence of changes occurring in pH and ionic composition which occurs in biological samples taken postmortem were investigated by comparing normal physiological conditions with a buffer system differing in pH, Na^+ and K^+ concentrations. DPBS (for amphetamine) or PBS (for amitriptyline) was used to represent the physiological condition. A 10 mM phosphate buffer (as in PBS) at pH 5.5 with 120 mM Na^+ and 25 mM K^+ represented the altered conditions. The pH was chosen as it is the common pH of blood within 24 hours after death (*Donaldson & Lamont 2013, Sawyer et al. 1988*), the changes in ionic concentrations represent the average ionic concentrations after death, as reported by (*Singh et al. 2002*). Sorption of both amphetamine and amitriptyline was tested in both conditions at equilibrium. The effect of this lower pH alone was also tested using sorption isotherms at pH 5.5 (both compounds) and pH 3.5 (amitriptyline only). These solutions consisted of a 10 mM Na-phosphate buffer with 138 mM Na^+ and 3 mM K^+.

Sampling with C18/SCX fibers in the kinetic uptake phase

Sorption after pre-equilibrium exposure times of 5 and 30 minutes for amphetamine and 30 minutes for amitriptyline (all on the roller mixer) were compared to sorption isotherms at equilibrium. Amphetamine was exposed in DPBS (pH 7.4, with 0.9 mM Ca^{2+}), whereas amitriptyline was exposed in PBS (pH 7.4). Figure S4 in the Supporting Information shows that this very low concentration of Ca^{2+} does not significantly influence sorption to the fiber, so any results in either DPBS or PBS can be directly compared.

Influence of agitation conditions on sorption kinetics to C18/SCX fibers

In *in vivo* studies and in studies with cells or proteins, (strong) agitation of the sample might not always be possible because agitation may simply not be feasible or agitation may affect the matrix. As uptake kinetics are essential to calibrate the SPME fiber, especially in pre-equilibrium sampling, the influence of agitation conditions on sorption kinetics were tested. Sorption isotherms at equal exposure times were made to investigate the influence of agitation conditions by comparing agitated or static sampling. For this, amphetamine was sampled from DPBS statically for 30 minutes and 5 minutes. Amitriptyline was sampled statically from PBS for 30 minutes.

Influence of temperature on sorption kinetics and affinity to C18/SCX fibers

Temperature of the sample and especially temperature of postmortem tissue can range between 37°C (body temperature) and 4°C (storage temperature). Uptake kinetics and sorption isotherms at equilibrium were made for amphetamine and amitriptyline, comparing sorption at 4°C, 20°C and 37°C. Exposure solution was PBS at pH 7.4 for both compounds. For these experiments, samples were agitated by placing them on an orbital shaker at 300 rpm instead of a roller mixer. Due to the size of the roller mixer, it could not be used in the stove at 37°C, so for a direct comparison all samples of the temperature experiments were agitated using the orbital shaker. Stability of amphetamine and amitriptyline was checked at 37°C, both did not show any degradation for the duration of the experiments (data not shown).

Competition effects on sorption to C18/SCX fibers in binary mixtures of basic drugs

To investigate a potential competitive effect within mixtures, different compositions of an amphetamine – amitriptyline mixture in PBS were made. Amphetamine concentrations ranging between 0.36 and 74 µM were prepared in 20 mL of PBS. To these concentrations, a fixed concentration of amitriptyline was added. The range of amphetamine concentrations was tested with four fixed amitriptyline concentrations: 7 nM, 90 nM, 900 nM or 9000 nM (named mixture 1 through 4). Fibers were exposed until equilibrium for amitriptyline, i.e. 3 days. Sorption to the C18/SCX fiber was also tested after a pre-equilibrium exposure of 30 minutes to mixture 4 (with 9000 nM amitriptyline).

HPLC and LC-MS analysis

The LC system used was a Prominence HPLC, consisting of two pumps, an auto sampler, a column oven and a UV-detector, all from Shimadzu ('s-Hertogenbosch, The Netherlands), and a C18 column (GraceSmart RP18, ID 150 x 2.1 mm, 5 µm particle size, Grace, Breda, The Netherlands). Column oven was set at 40°C, mobile phase flow rate was 0.4 ml/min. Mobile phase always consisted of a 10 mM phosphate buffer at pH 3 and acetonitrile. For amphetamine, mobile phase was pre-mixed at a ratio of 95:5 (buffer : acetonitrile). For amitriptyline, diclofenac and trimethoprim, mobile phase was mixed on-line at a ratio of 65:35, 55:45 or 85:15, respectively. UV detection wavelength was 208 nm for amphetamine, 239 nm for amitriptyline, 276 nm for diclofenac and 240 nm for trimethoprim. Limit of quantification (LOQ) was

set at a signal to noise ratio of 10:1 and was 350 nM for amphetamine, 60 nM for amitriptyline, 250 nM for diclofenac and 200 nM for trimethoprim. For the analysis of the mixtures of amphetamine and amitriptyline, UV detection was set at 208 nm for both compounds and the isocratic methods were replaced by a gradient. This gradient started at 5% acetonitrile for 1 minute, then increased to 40% acetonitrile in 1 minute and remained there for 2 minutes. At 4 minutes, the percentage acetonitrile was instantly decreased to 5% acetonitrile until the end of the run (7 minutes). Retention time of amphetamine was 3.2 minutes, and 4.6 minutes for amitriptyline.

Only for amitriptyline at very low aqueous concentrations, quantification was not possible using HPLC-UV as the aqueous concentrations are well below the LOQ of this method. To still establish a sorption isotherm with these concentrations, we analyzed these samples using LC-MS/MS. A Perkin Elmer liquid chromatography system (Norwalk, CT) was coupled to a triple quadrupole/linear ion trap mass spectrometer (MDS Sciex API 3000 LC-MS/MS System, Applied Biosystems, Foster City, CA). The interface was a Turbo Ion spray source set in positive ionization mode at 2000 V and operated at 400°C. Separation occurred through a Kinetex 2.6 µm XB-C18 column (50x2.1 mm, 100Å) with a UPLC C18 guard column. Mobile phase consisted of Milli-Q and methanol, both with added 0.1% formic acid. Each run was 12 minutes with the first 5 minutes at a flow rate of 200 µL/min with mobile phase at a ratio of 95:5 (MQ : MeOH), then increasing to 250 µL/min in 1 minute while changing to a mobile phase ratio of 1.5:98.5. This was maintained for 4.5 minutes, then returning to the initial flow rate and mobile phase ratio until the end of the run. A solvent switch was used to prevent nonvolatile salts from entering the MS, this was set to waste for the first 7 minutes and last 1.5 minutes of the run. Retention time of amitriptyline was 8.5 minutes. SPME extracts analyzed using LC-MS/MS were acidified using 1% formic acid (instead of 0.1 M HCl). Acquisition was achieved using Analyst 1.4.2 (MDS Sciex Applied Biosystems) in multiple reaction monitoring mode with fragmentation of the molecular ions at m/z 278.4/117.0. LOQ is 1 nM.

Quantification

For all SPME experiments, amphetamine was sampled from exposure solutions with a volume of 1.5 mL. For amitriptyline, SPME fiber exposure in a volume of 1.5 mL led to a large depletion of the aqueous phase of up to 90%, which influences sorption kinetics (*Oomen et al. 2000*). Therefore, all samples containing amitriptyline were exposed in a volume of 35 mL to

decrease depletion (<50%). This means that depletion is not always negligible but is accounted for in the calculation of the sorption coefficient by plotting the aqueous sample concentration remaining after exposure to the fiber instead of the initial sample concentration. Both fiber concentrations as well as aqueous concentrations were always measured. A calibration curve made from aqueous concentrations before exposure was used to calculate the remaining aqueous concentration after exposure and calculate depletion. A calibration curve in desorption fluid (acidified for experiments with the C18/SCX fiber) was used to calculate the fiber concentrations and check mass balances.

Data analysis

Data was plotted and analyzed using Graph Pad Prism 6 for Windows. Each sample was prepared in triplicate, unless otherwise specified. These are plotted as mean ± standard deviation in both x- (measured aqueous concentrations after fiber exposure) and y-direction (measured fiber concentrations).

RESULTS AND DISCUSSION

Comparison of the C18/SCX and C18 coating and fiber selection

Previous work on the C18/SCX fiber showed an almost equal sorption affinity of both the cationic and the neutral species of amphetamine, and demonstrated a much higher yield of this SPME coating compared to PA coatings (*Peltenburg et al. 2013*). However, this study only provided a limited comparison between the prototype C18/SCX coating and the commercially available biocompatible C18 coating. This C18 fiber has the same coating material as the C18/SCX fiber except for the embedded SCX groups. Since we initially assumed the SCX groups provided the main ion-exchange sorption sites for charged amphetamine, a detailed comparison with C18 would render information about the true cation-exchange capacity of the "mixed-mode" fiber. Therefore, the sorption affinity of amphetamine, amitriptyline and trimethoprim to both coatings was compared over a wide range of pH values (figure 1).

According to the manufacturer, the working pH range of C18-coated SPME fibers is 1-9, but prolonged exposure (>2 hours) to pH >7.5 could slowly damage the silica. For both the C18/SCX and the C18 fiber and for each of the three compounds, there is no large effect of pH on the sorption affinity (log D_{fw}) within this working pH range. There is a small increase in sorption affinity

for each compound between pH 2 and pH 7 (±0.5 log units), which is more pronounced for the C18/SCX fiber than for the C18 fiber. This observation could be explained by competition of H^+ for the strong cation exchange groups in the C18/SCX fiber, as previously suggested (*Peltenburg et al. 2013*).

For a comparison of D_{fw} values of the cationic and neutral species, pH values above the pKa values of the compounds should be included. This includes pH values outside of the working pH range of the fibers. We therefore studied coating degradation at high pH by exposing C18/SCX fibers to high pH (pH>8). This showed no coating degradation after 24 hours, but a significant coating degradation of 40% after 72 hours (Supporting Information, figure S5). For amphetamine and trimethoprim, which were exposed for 24 hours, direct comparison of the cationic and neutral fraction is therefore possible. For amphetamine, the difference between sorption affinity of the cationic and neutral fraction is 0.2 log units for the C18/SCX fiber and 0.4 log units for the C18 fiber (pH 7.4 vs pH 10.4). For a true sorption affinity of the neutral fraction of amphetamine, sorption data above pH 11 is necessary as the pKa of amphetamine is 9.9. Trimethoprim, however, has a pKa of 7.2, and is completely neutral at pH>9. For trimethoprim, the difference in sorption affinity of cationic and neutral fraction is 0.05 log units for the C18/SCX fiber (pH 4.3 vs pH 9.3) and 0.8 log units for the C18 fiber (pH 4.3 vs pH 9.0). Sorption affinity of trimethoprim was also determined using exposure times of 72 hours. Supporting Information, figure S6 compares trimethoprim sorption after 24 hour and 72 hour exposures for both fibers, showing similar sorption affinities between pH 2 and 7 (as equilibrium is reached <24 hours) and a decrease in sorption affinity at 72 hours compared to 24 hours (due to coating degradation). For amitriptyline, with a pKa of 9.4, the sorption affinity of the neutral compound again has to be determined above pH 11. Moreover, as fibers are exposed to amitriptyline for 72 hours to reach equilibrium, high pH damages the coating and therefore decreases calculated distribution coefficients. As the coating degradation at high pH is very consistent for each fiber (40% loss of sorption capacity), the measured sorption affinity can be corrected for this loss of phase. Figure 1 therefore shows the measured sorption affinity of amitriptyline, with an extrapolated sorption affinity based on 40% degradation (data in grey). Please note that coating degradation was only determined for the C18/SCX fiber, but is assumed to be similar for the C18 fiber. Although presenting data of amitriptyline at high pH shows some uncertainty and this data should not be used for calculations, it is clear that sorption of the neutral fraction of amitriptyline is not orders of magnitude

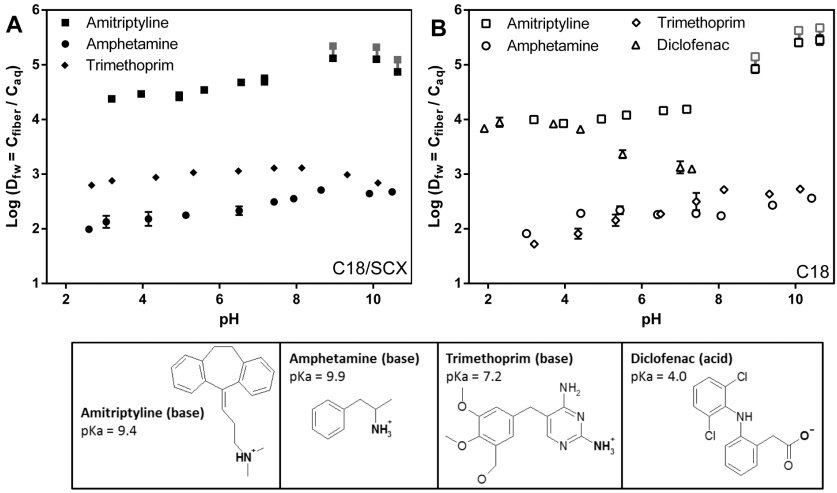

Figure 1. Sorption of the bases amphetamine, amitriptyline and trimethoprim and the acid diclofenac over a wide pH range to the C18/SCX and C18 fiber. Exposure solutions consisted of 10 mM buffers with 140 mM Na^+. For amphetamine and trimethoprim, fibers are exposed for 24 hours, while this is 72 hours for amitriptyline and diclofenac. For amitriptyline, data at high pH contains some uncertainty as coating degradation occurs after 72h, but not after 24h (see Supporting Information, figure S5). Coating degradation is consistently 40% for an exposure time of 72h. Black symbols indicate measured data, while grey symbols are extrapolated data based on coating degradation. Amphetamine concentration in all samples was 7.4 µM, amitriptyline concentration was 0.36 µM, trimethoprim concentration was 3.4 µM and diclofenac concentration was 1.7 µM. All samples were agitated at 40 rpm during exposure until equilibrium. All data points for the C18/SCX fiber are triplicate averages ± sd with exception of trimethoprim which is in duplicate. All data points for the C18 fiber are duplicates. pKa values are from (*ChemAxon, www.chemicalize.org*). Amphetamine data for the C18/SCX fiber is reprinted with publisher permission from (*Peltenburg et al. 2013*).

higher than sorption of the cationic fraction.

For almost completely ionized chemicals, it has been argued that the sorption affinity could represent only the neutral species (*Risticevic et al. 2010*). It is highly unlikely that this is the case in the current test systems as this would result in a large difference in sorption affinity at different pH between the ionized and neutral species, as was previously shown for amphetamine sorption to neutral PA fibers (*Peltenburg et al. 2013*). Here, the absence of such a pH effect clearly shows that the D_{fw} values represent sorption of the ionized species. pH-dependent sorption has in the past led to the hypothesis that only the undissociated form of the compound has sorption affinity to the coating, although this is believed to only be the case for absorptive coatings or extraction processes that are governed by non-polar interactions (*Lord & Bojko 2012)*. Theoretically, sorption of the neutral fraction to the fiber coating alters the equilibrium between dissociated and undissociated fraction as the sorbed compound is no longer available within this equilibrium (*Lord & Bojko 2012*). However, when comparing sorption affinities of cationic compounds over a pH range 2-7 (figure 1), the test compounds show small differences with increasing pH of around 0.5 log units from pH 2 to pH 7. At pH 7, the neutral fraction of amphetamine or amitriptyline is around 1%, and potential re-association of dissociated compound could restore the sorbed neutral fraction. However, at for instance pH 3, the neutral fraction is around 0.0001%, making it very unlikely that sorption at this pH is only dictated by the neutral fraction.

As previously described (*Peltenburg et al. 2013*), deprotonation of the silanol groups in the fiber coating could explain why the C18 fiber shows similar or only slightly lower sorption affinity for amphetamine and amitriptyline compared to the C18/SCX fiber. The SCX phase is supposed to interact via cation exchange. The same could also occur with negatively charged silanol groups. As these silanol groups have a reported pKa of 6.8 (*Escher et al. 2000*), they will become increasingly negatively charged at pH>5, thereby possibly adding to the maximum sorption capacity of the C18/SCX fiber, but also to the sorption capacity of the C18 fiber because there, the C18 functional groups are also attached to silica particles. As there is still sorption of cationic compounds to the C18 fiber at pH<3, where the silanol groups are not charged, this would imply that also charged organic compounds can sorb to C18 functional groups. To test whether the C18 material itself can sorb ionic compounds, this C18 fiber was used to test the pH-dependent sorption of diclofenac. Diclofenac is an acid

(pKa = 4.0 (*ChemAxon, www.chemicalize.org*)), and therefore completely negatively charged at pH>6. As the data in figure 1 show that there is still considerable sorption of diclofenac at pH 6 and higher, the anionic species is not specifically repulsed from the surface as would be expected in the presence of dissociated silanol groups. It may be that the large surface of the C18 coating on the porous support material itself acts as the main adsorbing phase for ionic compounds, and that neither the silanol groups nor the SCX groups appear to strongly contribute to the sorption affinity of the 3 tested drugs to these two C18-based SPME coatings. Recent studies on a diverse set of C18 HPLC columns (*Loeser 2008, Marchand & Snyder 2008*), however, indicated the presence of significant amounts of anion exchange sites that, specifically at low pH, retained anions as small as Cl^- and NO_3^-. So in addition to C18 surface interactions and attraction of cations by dissociated silanol groups, there may be positively charged sites on these C18 silica-based SPME coatings that interact favorably with anionic species as well. As adsorption to the C18 coating may imply a finite number of surface sites to be occupied for neutral compounds, or even an accumulation of charge for ionic species leading to repulsion, adsorption is a competitive sorption process that will result in a non-linear relationship between sorbed and dissolved concentrations at relatively high fiber loadings, as shown in figure 3. Also shown in figure 3 is that the adsorption process can approximate to a linear relationship at sufficiently low concentrations (*Górecki et al. 1999*).

For the three basic compounds tested, sorption affinity to the C18/SCX fiber is somewhat higher than to the C18 fiber. For amphetamine, the logarithmic value of the distribution coefficient (log D_{fw}) for the C18/SCX fiber at pH 7.4 is 2.49±0.02 and 2.28±0.04 for the C18 fiber at pH 7.4. For amitriptyline, log D_{fw} for the C18/SCX fiber at pH 7.4 is 4.72±0.10 and 4.19±0.05 for the C18 fiber at pH 7.4. For trimethoprim, this is 3.02±0.01 for the C18/SCX fiber at pH 7.4 and 2.38±0.16 for the C18 fiber at pH 7.4. (data from figure 1). The slightly higher D_{fw} values of these compounds for the C18/SCX fiber are most likely the result of additional sorption of the cation species to the strong cation exchange groups. This is also in line with previous findings that positively charged electrolytes such as Na^+ and K^+ show competition for the ion-exchange sites in the C18/SCX coating (*Peltenburg et al. 2013*). Between the C18 and C18/SCX fiber only a small difference in sorption affinities exists for the tested compounds. For cationic compounds that show high sorption affinity to both fibers, this may even present an advantage of using C18 coatings, because these may be less prone to the competitive effects of salts as demonstrated for C18/SCX

(*Peltenburg et al. 2013*). Because C18/SCX fibers have been recommended for the simultaneous sorption of different metabolites (log K_{ow} ranging between -3 and 7) (*Vuckovic & Pawliszyn 2011*) in *in vivo* metabolomics profiling studies (*Bojko et al. 2013, Cudjoe et al. 2013, Vuckovic et al. 2011a*), and in order to further examine influence of system variables on the prototype C18/SCX fiber for the extraction of basic drugs, the focus of the current study remains on the C18/SCX coating.

The difference in D_{fw} between the three cationic compounds can be explained by their difference in hydrophobicity. Neutral amphetamine and neutral trimethoprim have a log K_{ow} of 1.80 and 1.28, respectively, whereas this is orders of magnitude higher for neutral amitriptyline (log K_{ow} = 4.81) (*ChemAxon, www.chemicalize.org*). Sorption affinity of these compounds for the C18 fiber follows this order of hydrophobicity, indicating that the hydrophobic groups within the compound govern sorption to the fiber. For the C18/SCX fiber, trimethoprim has a higher sorption affinity than amphetamine. This could be explained by a difference in the interaction of the compounds with the coating as the C18/SCX fiber is expected to have more than one sorption mechanism. Possibly, size or presence of other polar groups within the molecule contribute to the overall sorption affinity of a compound.

Our previous paper reported a maximum sorption capacity of the C18/SCX fiber of 47 mmol/L coating (*Peltenburg et al. 2013*) and this value was based on amphetamine sorption data at aqueous concentrations above 1 mM. However, when including the current data on amitriptyline, this maximum sorption capacity appears to be even higher, as can be seen in figure 3. At the highest aqueous concentration tested, amitriptyline fiber concentrations are around 125 mmol/L coating. As sorption levels off at high aqueous concentrations as all surface sites become occupied, this results in a combined maximum sorption of the compound to the surface of the C18 chains and the negatively charged SCX groups. Compounds with relatively high sorption affinities readily deplete their surrounding system if sample volumes are limited, which is why in further experiments, we used different sample volumes for amphetamine and amitriptyline (1.5 and 35 mL, respectively). Decreasing the fiber volume by cutting the fiber would result in a lower sensitivity, and was considered to be too inaccurate, as a precise coating volume is needed to normalize the amount of drug sorbed to the fiber. Furthermore, as the relative amount of drug that has to be transported to the SPME coating via diffusion is much higher for amitriptyline than for amphetamine because of its higher distribution

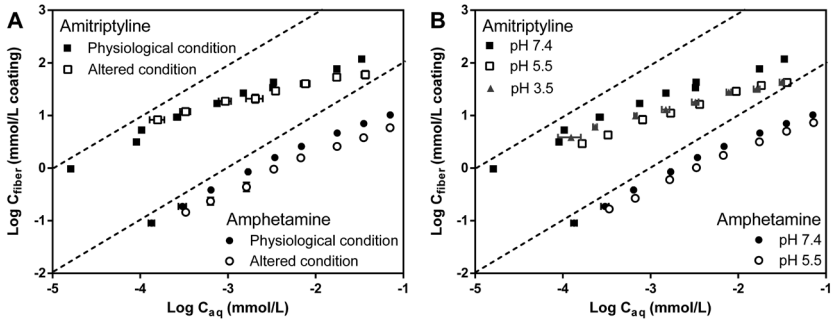

Figure 2. Sorption isotherms for the C18/SCX fiber comparing normal physiological condition and the altered condition (A) and different pH (B). DPBS (amphetamine) or PBS (amitriptyline) represents the normal physiological condition. The altered condition was a 10 mM phosphate buffer (as in PBS) with 120 mM Na^+ and 25 mM K^+. Buffers at pH 5.5 and pH 3.5 consisted of a 10 mM Na-phosphate buffer with 138 mM Na^+ and 3 mM K^+. All samples were agitated at 40 rpm during exposure until equilibrium. Data was measured in triplicate and plotted as mean ± sd. Dashed lines indicate linearity.

coefficient, this results in a much longer equilibration time for amitriptyline compared to amphetamine (see Supporting Information, figure S3).

Investigating the effects of different test conditions for sampling with C18/SCX fibers

Influence of pH and ionic composition on SPME affinity

To study the influence of changes in pH and ionic composition on sorption to the C18/SCX fiber, sorption isotherms of amphetamine and amitriptyline were made using different exposure solutions that mimic these changes (figure 2A). Sorption of both amitriptyline and amphetamine to the C18/SCX fiber is slightly decreased with ±0.2 log units for the condition at pH 5.5 and altered ionic composition compared to normal physiological condition. However, as both the pH and the ionic composition is altered, it is difficult to identify the variable that causes the decrease in sorption between these two conditions. To investigate this, additional sorption isotherms were made which differ only in their pH (figure 2B). The sorption affinity at pH 5.5 and in the altered condition (also pH 5.5, and a changed ionic composition) are comparably decreased to the normal physiological condition. This shows that the decrease

in sorption between the two conditions is not caused by the difference in ionic composition but by the difference in pH. High concentrations of K^+ (140 mM) previously showed a large decrease in the sorption affinity of amphetamine for the C18/SCX fiber compared to buffer only (*Peltenburg et al. 2013*). The K^+ concentration used here is much lower (25 mM) and the ionic strength of the two conditions is equal, explaining why the decrease in sorption affinity is not caused by the slight increase in K^+ concentration compared to normal physiological conditions.

When comparing the sorption isotherm at pH 5.5 to that at pH 7.4 (figure 2B), the sorption affinity is slightly decreased at lower pH. Sorption of amitriptyline was also studied at pH 3.5. Sorption at pH 3.5 is similar to the sorption isotherm at pH 5.5, indicating that the concentrations of H^+ do not affect the sorption affinity at pH 3.5, 5.5 and 7.4. As mentioned in paragraph 3.1, deprotonation of silanol groups at pH>5 possibly adds to the maximum sorption at pH 7.4 but not at pH 3.5 and 5.5. Also, the contribution of the strong cation exchange groups in the C18/SCX fiber to sorption is most likely small, and the competitive effect of positively charged electrolytes is therefore indeed also limited, and will be compensated for when SPME calibration is performed in appropriate medium. As discussed above, adsorption to the C18 surface is probably the dominating sorption process for ionic compounds, but the minor salinity effects observed here and in our earlier work with C18/SCX (*Peltenburg et al. 2013*) may be due to the effect of salts on the electrostatic double layer caused by the SCX groups. Although it remains to be tested, biocompatible C18 SPME may therefore be even less susceptible to salinity effects.

Sampling with C18/SCX fibers in the kinetic uptake phase

The time needed to reach equilibrium between the aqueous phase and the C18/SCX fiber coating can be relatively long for compounds with a high affinity to the C18/SCX coating, because a larger amount has to be transported from the aqueous phase into the fiber coating. Equilibrium SPME sampling typically results in the most accurate determination of the concentration, because it is, for example, independent of the diffusion kinetics in different matrices. However, the application of SPME sampling after short exposure times will have practical advantages for example in *in vivo* or in vitro studies. Moreover, in toxicokinetic studies, exposure times must sometimes be short enough so that the sampling does not interfere with the change in concentration in blood or tissue. Studying sorption at pre-equilibrium (in the kinetic uptake phase) greatly improves the practicality of SPME sampling.

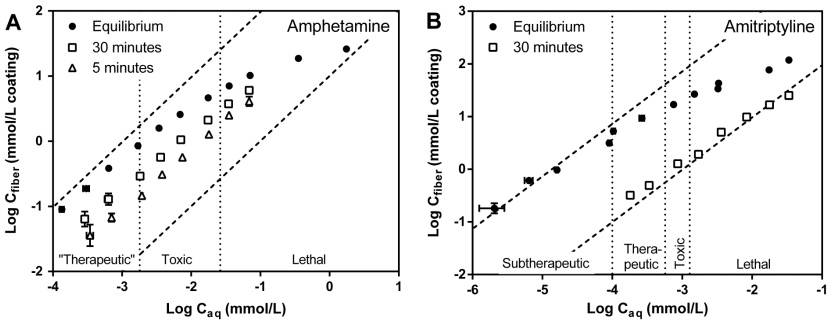

Figure 3. Sorption isotherms to C18/SCX fiber for equilibrium and pre-equilibrium agitated sampling of amphetamine (A) and amitriptyline (B). Exposure solutions consisted of DPBS (amphetamine) or PBS (amitriptyline), both at pH 7.4. Samples were agitated at 40 rpm on a roller mixer during exposure. Equilibrium exposure time is 2 hours for amphetamine and 3 days for amitriptyline (see Figure S3). For amitriptyline, aqueous concentrations below log C_{aq} of -4 (0.1 µmol/L) were measured using LC-MS/MS (see method section). Data was measured in triplicate and plotted as mean ± sd. Dashed lines indicate linearity. Please note the difference in x- and y-axis between the figures. Amphetamine data at equilibrium is reprinted with publisher permission from (*Peltenburg et al. 2013*).

As expected, shorter exposure times lead to lower fiber concentrations at equal aqueous concentrations (figure 3A & 3B). For all exposure times, fiber concentrations tended to level off at higher aqueous concentrations. Sorption data from experiments in the kinetic phase follow the dashed lines with a slope of 1 on a logarithmic scale, indicating that sorption is a linear process in the tested concentration ranges. Sampling in the kinetic uptake phase leads to more linear isotherms because sorption approaches linearity at the lower fiber concentrations that are reached at pre-equilibrium sampling. Extraction recoveries of amitriptyline and amphetamine differ with exposure time (equilibrium vs. pre-equilibrium) and with different concentrations (because of the nonlinearity at high aqueous concentrations). Table S1 in the Supporting Information shows extraction recoveries for different concentrations and exposure times for amphetamine and amitriptyline (as taken from figure 3).

Obviously, very short exposure times could also lead to decreased analytical sensitivity as fiber concentrations drop below LOQ within the tested aqueous

concentration range. In figure 3A, the averaged relative standard deviation between the triplicates is 6% after equilibrium extraction, and 15% after a 5 minute extraction, showing that shortening the exposure time leads to an increased spread across the triplicates. When exposing the fiber in the kinetic uptake phase, the exact exposure time should be chosen carefully so that the method is practical, yet not suffering a loss in sensitivity. However, as the C18/SCX fiber displays high D_{fw} values for the compounds tested here, decreasing the exposure time does not lead to a loss in sensitivity, making it a rapid and sensitive sampling tool.

Influence of agitation conditions on sorption kinetics to C18/SCX fibers

For the future practical application of pre-equilibrium SPME in samples from for example *in vivo*, toxicokinetic and forensic studies, agitation conditions were investigated as agitation might not always be possible. Uptake kinetics of amphetamine and amitriptyline were shown to be considerably slower for static sampling compared to agitated sampling, increasing equilibration time for amphetamine from 1.2 hours to 3.4 hours and for amitriptyline from 1.3 days to 15 days (Supporting Information, figure S3). Sorption isotherms derived from static pre-equilibrium exposures were compared with pre-equilibrium exposures with agitation (figure 4). As the uptake kinetics are slower for static sampling compared to agitated sampling, equal pre-equilibrium exposure times yield higher fiber concentrations in experiments with agitation.

If diffusion in the aqueous phase or in the aqueous diffusion layer is rate-limiting, agitation of the sample may lead to higher uptake rates and shorter equilibration times (*Pawliszyn 2012*). The data in figure S3 show that equilibration is faster in the experiments with agitation of the SPME fibers. This shows that diffusion in the aqueous phase is rate-limiting. For SPME fibers with C18 functional groups, it is known that kinetics to these porous fibers depend mainly on mass transfer in the unstirred boundary layer and that also extraction rates are highly influenced by the degree of agitation (*Liu et al. 1997*).

Influence of temperature on sorption kinetics and affinity to C18/SCX fibers

To be able to apply the current SPME set-up in *in vivo* sampling, calibration in an appropriate medium and in the correct environmental condition is crucial. Temperature is another factor that influences sorption to the fiber and should be considered when applying the fiber *in vivo*. Temperature after death drops

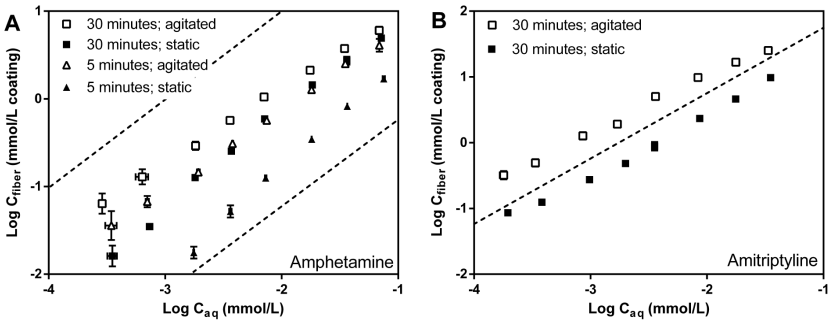

Figure 4. Sorption isotherms for C18/SCX fiber at pre-equilibrium in agitated and static conditions for amphetamine (A) and amitriptyline (B). Exposure solutions were DPBS for amphetamine and PBS for amitriptyline, both at pH 7.4. Exposure times are 30 minutes (squares) and 5 minutes (triangles; for amphetamine only). The agitated samples were placed on a roller mixer at 40 rpm during exposure. Data was measured in triplicate and plotted as mean ± sd. Dashed lines indicate linearity. Please note the difference in y-axis between the figures.

from body temperature (37°C) to, eventually, storage temperature (often 4°C). Figure S7 in the Supporting Information shows the influence of different temperatures on the uptake kinetics of amphetamine and amitriptyline to the C18/SCX fiber. In general, an increase in temperature causes an increase in the uptake rate, due to an increase in diffusion coefficients, and a decrease in sorption affinity, due to an increase in solubility of the analyte (*Pawliszyn 2012*). Figure S7, however, shows an almost negligible influence of temperature on the sorption isotherms of amphetamine and amitriptyline at equilibrium. For amphetamine, logarithmic D_{fw} values (at C_{aq} = 1 µM) are 2.77 to 2.75 to 2.76 at 4°C, 20°C and 37°C, respectively. For amitriptyline, log D_{fw} ranges from 4.17 to 4.27 to 4.09, respectively, with increasing temperature. Temperature-dependent sorption of poly-aromatic hydrocarbons to PDMS coated SPME fibers showed comparably minor decreases in sorption affinity over a comparable temperature range (*Muijs & Jonker 2009*). As with the previously described salinity effect, the effect of the operational temperature range on sorption affinity is marginal for equilibrated application of C18/SCX SPME, but pre-equilibrium sampling should be carefully calibrated.

Competition effects on sorption to C18/SCX fibers in binary mixtures of basic drugs

Mixtures of amphetamine and amitriptyline were used to gain insight into potential competition effects within mixtures. It is important to note that any mixture of these two compounds is not clinically relevant as the combination is hardly seen, but to obtain a more general insight into competition effects, it is very relevant. Moreover, competition effects for the C18/SCX fiber are expected to be based on the same mechanism for any combination of two cationic compounds.

The effect of a constant amitriptyline concentration on the sorption of a range of amphetamine concentrations was tested using four different amitriptyline concentrations (figure 5). In figure 5A, the mixtures with the highest concentrations of amitriptyline (mixtures 3 with 900 nM and mixture 4 with 9000 nM amitriptyline) cause a decrease in amphetamine fiber concentrations in a concentration dependent manner, at maximum by a factor of 15. For mixture 4 (9000 nM), fiber concentrations belonging to the lowest tested amphetamine concentration dropped below the LOQ. The two lowest amitriptyline concentrations in the mixtures (7 nM and 90 nM) did not decrease sorption of amphetamine.

Figure 6 shows the total fiber loading of the different mixtures. As described in paragraph 3.2.2, the maximum sorption of the C18/SCX fiber is around 125 mmol/L. Sorption is linear up to 10 mmol/L, as can be deduced from the individual sorption isotherm in figure 3. For mixtures 1 and 2, the total fiber loading is mostly well below this 10 mmol/L. It appears that within this linear sorption range, competition effects do not significantly influence sorption to C18/SCX fibers at equilibrium. For mixtures 3 and 4 (at equilibrium), the total fiber loading is in the nonlinear range and approaching the maximum fiber loading. This results in a decrease in amphetamine sorption affinity, even though at most aqueous concentrations, amphetamine loading is well below 10 mmol/L. Sorption of mixtures of two cationic surfactants to ion-exchange PA fibers was also shown to be non-competitive in the linear range of the sorption isotherm (up to 2 mmol/L coating), while competition did occur when the total fiber loading exceeded 2 mmol/L coating (*Chen et al. 2012*).

As the four mixture experiments described above were performed at equilibrium, it is interesting to expand this data with mixture sorption at

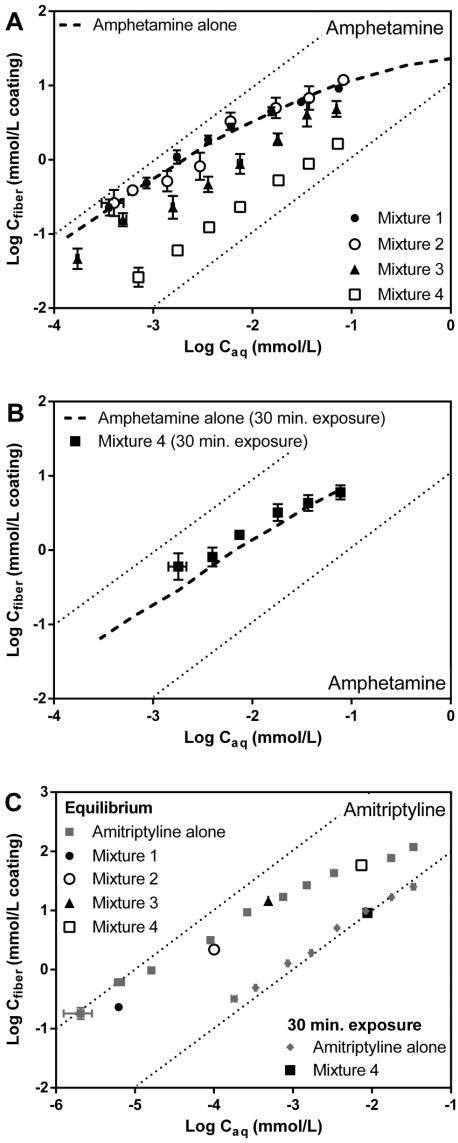

Figure 5. Competition effects within mixtures of amphetamine and amitriptyline. Four mixtures were tested, with variable amphetamine concentrations at constant ami-triptyline concentrations of 7 nM (mixture 1), 90 nM (mixture 2), 900 nM (mixture 3) and 9000 nM (mixture 4). All samples were agitated at 40 rpm during exposure until equilibrium. All data points are triplicates plotted as mean ± sd. Dotted lines indicate linearity. A) Amphetamine sorption to the C18/SCX fiber after equilibrium exposure (3 days) to the four mixtures. The dashed line represents the sorption of amphetamine as a single compound to the C18/SCX fiber at equilibrium. B) Amphetamine sorption from mixture 4 after an exposure of 30 minutes (kinetic uptake phase). The dashed line represents the sorption of amphetamine as a single compound to the C18/SCX fiber after a 30 minute exposure. C) Amitriptyline sorption for both equilibrium and pre-equilibrium sorption experiments. Grey data points represent sorption of amitriptyline as a single compound, the other data points represent amitriptyline sorption from the tested mixtures.

shorter exposure times (pre-equilibrium exposure). While mixture 4 showed significant competition at equilibrium, this is not the case for a 30 minute exposure (figure 5B). When comparing the total fiber loading after this 30 minute exposure to the same mixture after equilibrium exposure, the shorter exposure time leads to a fiber loading within the linear range of the fiber (figure 6), thereby explaining why competition is not observed. However, the two lowest amphetamine concentrations approached the LOQ and the calculated mass balance was below 50% compared to the other data points where the mass balance was always 90-110%, so these samples were not quantified.

As the sorption affinity of amitriptyline is around two orders of magnitude higher than that of amphetamine, the competitive effect appears to be limited to a decrease in amphetamine sorption only. Figure 5C shows the amitriptyline concentrations for the four mixtures after equilibrium exposure and the mixture after pre-equilibrium exposure compared to sorption of amitriptyline alone. No apparent decrease in amitriptyline fiber concentrations is seen for the mixtures compared to the single compound. The dominance of sorption by compounds with the highest affinities has for example also been observed for mixtures of nonionic surfactants, but is still dependent on the concentrations of each component relative to the sorption affinity (*Droge & Hermens 2010*). Calibration of the C18/SCX fiber in applied samples would only pose problems if the sum of all extracted compounds exceeds a fiber loading of 10 mmol/L after very short exposure times.

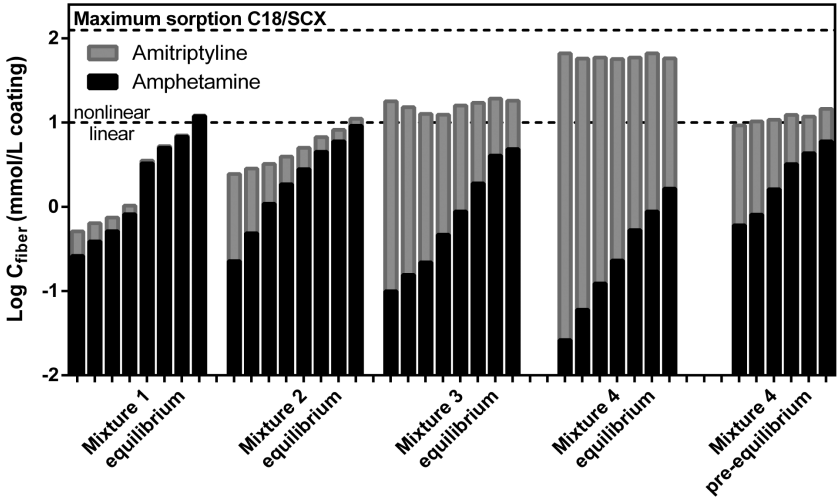

Figure 6. Total fiber loading of amphetamine and amitriptyline from mixture experiments to the C18/SCX fiber. Four mixtures were tested, with variable amphetamine concentrations at constant amitriptyline concentrations of 7 nM (mixture 1), 90 nM (mixture 2), 900 nM (mixture 3) and 9000 nM (mixture 4). All samples were agitated at 40 rpm during exposure. Data is plotted as the mean of a triplicate fiber exposure. The C18/SCX fiber was either exposed for 3 days (equilibrium) or for 30 minutes (pre-equilibrium). Maximum sorption is 125 mmol/L. Sorption is linear up to 10 mmol/L (see figure 3). Relative standard deviation between all amitriptyline fiber concentrations within each mixture (n=24) is 7% for mixture 1, 6% for mixture 2, 13% for mixture 3, 10% for mixture 4 at equilibrium and 10% for mixture 4 after a 30 minute exposure.

CONCLUSION

The current paper elucidates the potential sorption mechanism of cationic compounds to the SPME fibers with C18 and C18/SCX coatings. The C18/SCX coating sorbs both neutral and cationic species with almost equal affinity, whereas bulk polymer coatings sorb neutral compounds orders of magnitude more effectively. However, since also C18 coatings sorb cationic species almost as efficiently as neutral species, the SCX moiety does not seem to be the key sorption site in the C18/SCX coating. Also the role of negatively charged silanol groups on the silica support material of both the C18 and C18/SCX coating seems small, because even anionic diclofenac species sorbed strongly to the C18 SPME. Ionized organic species seem to be substantially adsorbed to the high surface area of C18 in SPME types using porous silica based coatings. The charged species of the relatively hydrophobic basic drug amitriptyline has a hundredfold higher affinity to C18/SCX than the cationic species of the small basic drug amphetamine, and also a much longer equilibration time (±2 days vs. ±1 hour).

The C18/SCX SPME fiber is useful as an equilibrated passive sampling tool, but can also be employed at (practically more relevant) short exposure times in the kinetic uptake phase for both amitriptyline and amphetamine. Although sorption affinity at equilibrium is independent of the agitation conditions, agitation of the sample strongly influences the kinetic sampling method. Also temperature of the sample during fiber exposure influences kinetic sampling, indicating the necessity of careful calibration. The effect of solid matrix components in actual tissues on kinetic sampling still needs to be evaluated for ionogenic compounds.

Competition between drugs affects sorption to the C18/SCX in a concentration dependent manner. Although the current experiments only included binary mixtures of two cationic compounds, the data show that competition only occurs at high total fiber coating loadings in the nonlinear range, where at least one of the tested drugs was present well above therapeutic concentrations.

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SUPPORTING INFORMATION

Desorption of amphetamine and amitriptyline from the C18/SCX fiber

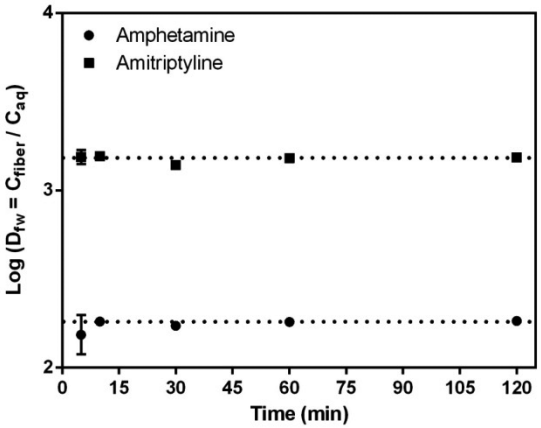

Figure S1. Desorption of both amphetamine and amitriptyline from the C18/SCX fiber was tested. Fibers were exposed for 2 hours to a concentration of 74 µM for amphetamine and 36 µM for amitriptyline. Exposure solution was DPBS for amphetamine and PBS for amitriptyline. Exposure volume was 1.5 mL for both compounds. After a 2 hour exposure, fibers were transferred to 120 µL of 90% acetonitrile and 10% MilliQ water with 0.1% NH_3 (end concentration). Different desorption times were tested. After this first desorption, all fibers were desorbed a second time for 2 hours. All desorption samples were acidified before HPLC injection with 60 µL 0.1 M HCl. Data was measured in triplicate and plotted as mean ± sd. Standard deviation is often not visible because of low variation in the data. From the results, it is concluded that a 15 minute desorption would be sufficient to completely desorb the fibers. The second desorption showed that carry-over was <4% for both compounds (not shown). The dotted lines serve to guide the eye. Amphetamine data is reprinted with publisher permission from the Supporting Information of ref. 1.

Reproducibility between C18/SCX fiber batches and repeatability when re-using fibers

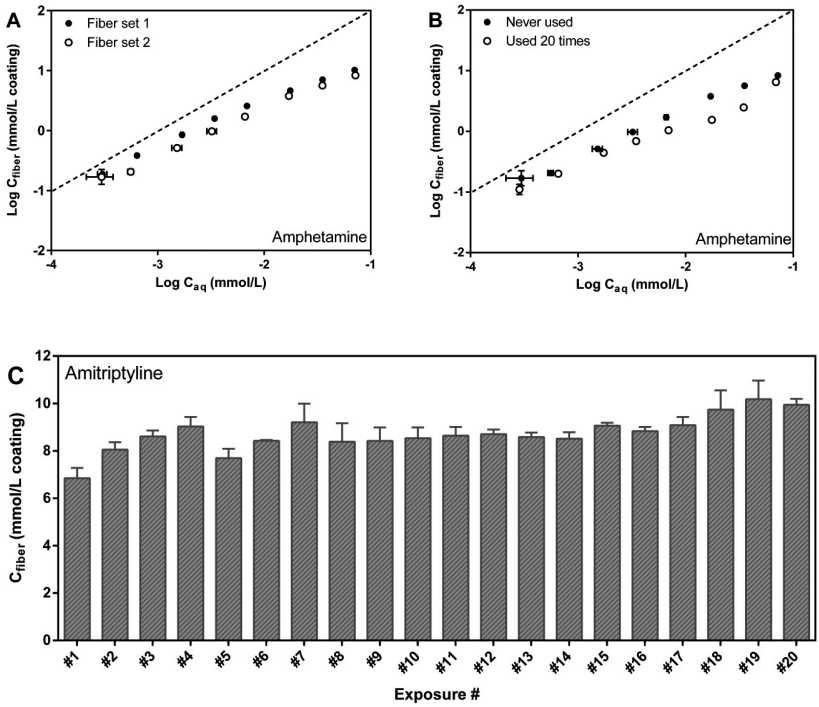

Figure S2. Sorption isotherms of amphetamine were made to test the reproducibility of the C18/SCX fibers. Exposure solution was DPBS (pH 7.4) and fibers were exposed at equilibrium (2 hours). Different sets of fibers were used to test inter-batch reproducibility (A), this proved to be highly reproducible. To test whether coating degradation occurred after re-use of the fibers, we tested the same fibers new and after 20 experiments (B). These fibers were only used for amphetamine. Most of the 20 experiments in between were sorption tests at pH 7.4, but 4 experiments were performed at pH 11.4. Sorption has somewhat decreased (0.1-0.4 log units), this could be due to coating degradation at high pH. To test whether sorption affinity decreases solely through coating degradation caused by re-use, we exposed 3 new fibers repeatedly to 3.6 µM amitriptyline in PBS for 30 minutes (C). Fibers were desorbed as usual, at least 15 minutes but usually longer and sometimes overnight. When not in use (not in PBS or in desorption fluid),

the fibers were stored in 50/50 MeOH/Milli-Q. Twenty consecutive exposures shows fiber concentrations ranging between 6.8 and 10.2 mmol/L coating. Relative standard deviation between all fiber concentration is 9.4%, average RSD of the twenty triplicates is 4.5%. Overnight desorption occurred between exposures #2 and #3, #4 and #5, #9 and #10, and #18 and #19 (the latter 66 hours). Data was measured in triplicate and plotted as mean ± sd. Standard deviation is often not visible because of low variation in the data. Inter-batch reproducibility data is reprinted with publisher permission from the Supporting Information of ref. 1.

Uptake curves for C18/SCX fiber for amphetamine and amitriptyline in agitated and static conditions

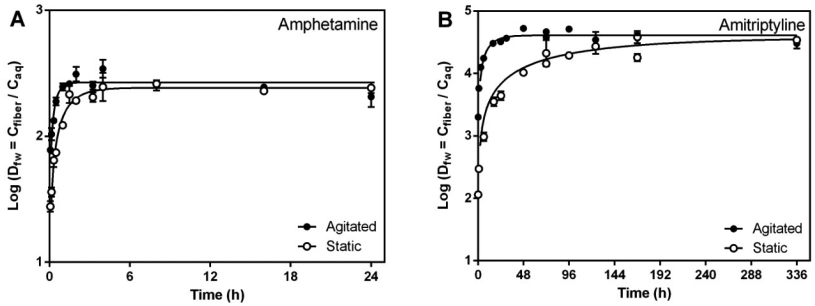

Figure S3. Uptake curves of amphetamine and amitriptyline to the C18/SCX fiber. Exposure solution was DPBS (amphetamine) or PBS (amitriptyline), both at pH 7.4. For both compounds, static sampling reaches the same distribution coefficient at equilibrium as agitated sampling but equilibration time is longer. For amphetamine, calculated equilibration time are 1.2 hours for agitated sampling and 3.4 hours for static sampling. For amitriptyline, the equilibration time for agitated sampling is 30 hours, while for static sampling, this is 362 hours (15 days). During experiments at equilibrium in agitated conditions, equilibration time for amphetamine was set at 2 hours and for amitriptyline at 3 days as this was more practical. Please note the difference in x- and y-axis between the figures. Data was measured in triplicate and plotted as mean ± sd. Standard deviation is often not visible because of low variation in the data. Amphetamine data of agitated sampling is reprinted with publisher permission from the Supporting Information of ref. 1.

Comparison of amitriptyline sorption from PBS and DPBS using the C18/SCX fiber

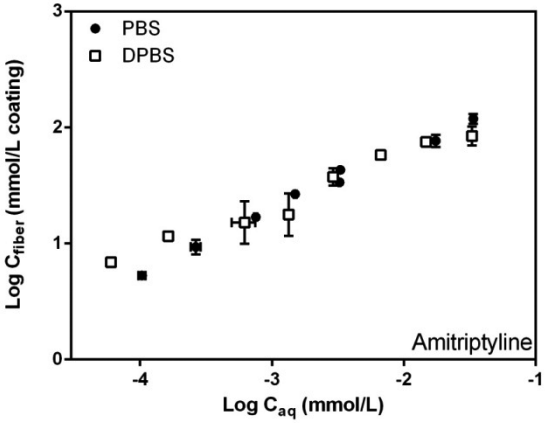

Figure S4. Sorption of amitriptyline from PBS and DPBS was compared to ensure no difference existed between the two solutions. The difference between PBS and DPBS is that the latter contains 0.9 mM Ca^{2+}. Amitriptyline was exposed to different concentrations in 35 mL of PBS or DPBS at equilibrium (3 days). Results are very similar, so the results of sorption from either PBS or DPBS can be directly compared. Data was measured in triplicate and plotted as mean ± sd. Standard deviation is often not visible because of low variation in the data.

Coating degradation of C18/SCX fibers at high pH

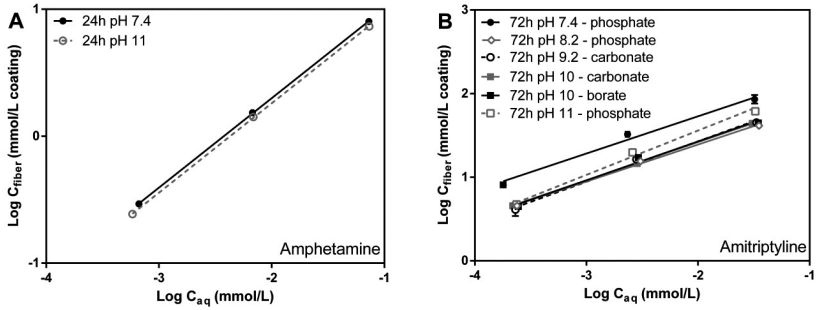

Figure S5. Coating degradation was checked at pH 9, pH 10 and pH 11 for amitriptyline and at pH 11 for amphetamine. Fibers were exposed for 24 hours (amphetamine) or 72 hours (amitriptyline). These fibers were then used to make a sorption isotherm in PBS (pH 7.4) of three concentrations (0.1; 1.0; 10 mg/L) in triplicate. Isotherms were fitted with the Freundlich isotherm. For amphetamine (A), a 24 hour exposure to pH 11 does not cause coating degradation. For amitriptyline (B), 72 hour exposures to pH 8, pH 9, pH 10 and pH 11 all showed some degree of coating degradation ($\pm 40\%$). The buffer used to set the pH does not appear to influence the degree of coating degradation. Relative standard deviation between the triplicates is not increased at high pH (6.1% at pH 7.4, 3.6% at pH 8.2, 8.0% at pH 9.2, 5.7% for pH 10 carbonate buffer, 4.3% for pH 10 borate buffer and 2.1% at pH 11), so that even the amount of coating degradation is highly reproducible. Amphetamine data is reprinted with publisher permission from the Supporting Information of ref. 1.

Sorption affinity of trimethoprim to C18 and C18/SCX fibers at different pH after 24 hours and 72 hours

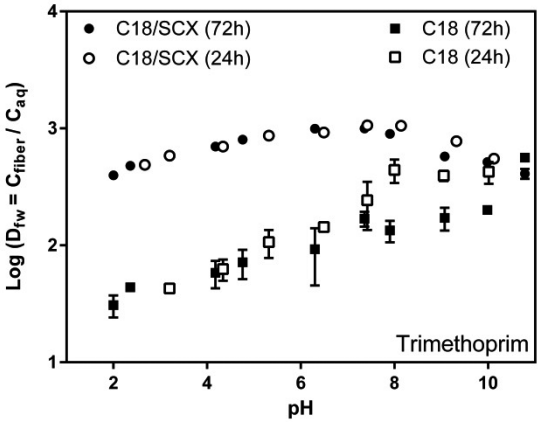

Figure S6. Sorption of trimethoprim was tested for both fibers at different pH, with two different exposure times. Exposure solutions consisted of 10 mM buffers with 140 mM Na^+, trimethoprim concentration was 3.4 µM. All samples were agitated at 40 rpm during exposure. All data points are duplicate measurements ± sd. For both fibers, sorption affinity between pH 2 and pH 7 is equal, as equilibrium has been reached <24 hours. From pH 8 and above, sorption affinity at 72h is decreased compared to sorption affinity at 24h, which is caused by degradation of the coating at high pH (in line with data in figure S5).

Temperature-dependent uptake kinetics to the C18/SCX fiber

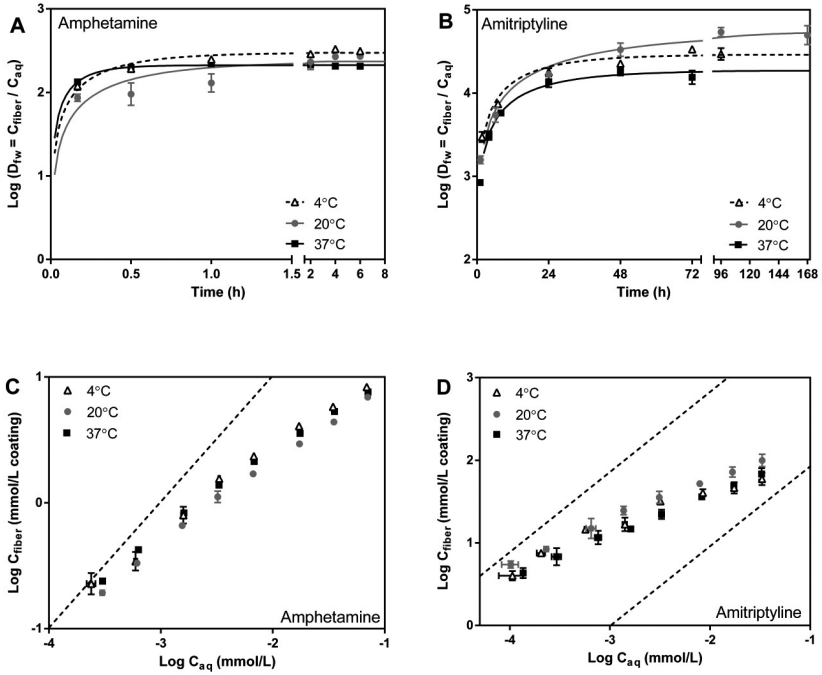

Figure S7. Uptake curves (A&B) and sorption isotherms (C&D) of amphetamine and amitriptyline were made at 4°C and 37°C and compared to sorption at 20°C. Exposure solution was PBS at pH 7.4 for both compounds. Samples were agitated using an orbital shaker at 300 rpm. Calculated equilibration times ($t_{95\%}$) are for amphetamine 1.1 h at 4°C, 1.6 h at 20°C and 0.5 h at 37°C and for amitriptyline 57 h at 4°C, 149 h at 20°C and 63 h at 37°C. Please note the difference in x- and y-axis between the figures. Data was measured in triplicate and plotted as mean ± sd. Standard deviation is often not visible because of low variation in the data.

Extraction recoveries of amitriptyline and amphetamine for the C18/SCX fiber

Amitriptyline (equilibrium)

Input amount (ng)	**Amount extracted (ng)**	**sd**	**Extraction recovery**	sd
35	26	7	75%	19%
180	89	14	49%	7,8%
349	141	9	40%	2,5%
1747	769	26	44%	1,5%
3493	1354	213	39%	6,1%
8733	2451	131	28%	1,5%
17465	3871	188	22%	1,1%
34930	4883	100	14%	0,3%
174650	11134	1406	6,4%	0,8%
349300	17196	1798	4,9%	0,5%

Amitriptyline (30 min exposure)

Input amount (ng)	Amount extracted (ng)	sd	Extraction recovery	sd
1747	46	7,2	2,7%	0,41%
3493	72	9,6	2,1%	0,28%
8733	184	25,0	2,1%	0,29%
17465	278	34,4	1,6%	0,20%
34930	731	22,4	2,1%	0,06%
84830	1424	159,3	1,7%	0,19%
174650	2430	59,7	1,4%	0,03%
349300	3662	232,2	1,0%	0,07%

Amphetamine (equilibrium)

Input amount (ng)	Amount extracted (ng)	sd	Extraction recovery	sd
37,5	6,4	0,7	17%	2,0%
75	13,2	1,4	18%	1,9%
150	27,2	3,2	18%	2,1%
375	60,1	3,4	16%	0,91%
750	112	6,6	15%	0,88%
1500	182	4,1	12%	0,27%
3750	328	10,6	8,7%	0,28%
7500	499	30,0	6,7%	0,40%
15000	724	25,2	4,8%	0,17%
75000	1320	79,7	1,8%	0,11%
375000	1845	68,3	0,5%	0,02%

Amphetamine (30 min exposure)

Input amount (ng)	Amount extracted (ng)	sd	Extraction recovery	sd
75	4,53	1,37	6,0%	1,8%
150	9,12	2,03	6,1%	1,4%
375	20,6	2,33	5,5%	0,6%
750	40,1	1,92	5,3%	0,3%
1500	74,3	1,80	5,0%	0,1%
3750	150	3,69	4,0%	0,1%
7500	266	7,52	3,5%	0,1%
15000	427	44,0	2,8%	0,3%

Amphetamine (5 min exposure)

Input amount (ng)	Amount extracted (ng)	sd	Extraction recovery	sd
75	2,53	1,18	3,4%	1,6%
150	4,75	0,76	3,2%	0,5%
375	10,3	0,66	2,8%	0,2%
750	21,9	2,09	2,9%	0,3%
1500	40,1	3,16	2,7%	0,2%
3750	90,1	6,86	2,4%	0,2%
7500	178	9,36	2,4%	0,1%
15000	289	52,2	1,9%	0,3%

Table S1. The input amount is the amount spiked to the vials before fiber exposure (ng). The amount extracted is the amount measured on the fiber using HPLC (ng), taken from a triplicate measurement and displayed as mean ± sd. Extraction recovery is calculated using amount extracted divided by the total input amount x 100%, standard deviation of the extraction recovery is calculated by dividing the standard deviation of the amount extracted by the total input amount x 100%. All data is from experiments displayed graphically in figure 3 of the manuscript.

1. H. Peltenburg, F.A. Groothuis, S.T.J. Droge, I.J. Bosman, J.L.M. Hermens, *Elucidating the sorption mechanism of "mixed-mode" SPME using the basic drug amphetamine as a model compound*, Analytica Chimica Acta (**2013**), 782, 21-27.

SORPTION OF STRUCTURALLY DIFFERENT PHARMACEUTICAL AND ILLICIT DRUGS TO A MIXED-MODE COATED MICROSAMPLER

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ABSTRACT

The mixed-mode (C18/strong cation exchange-SCX) solid-phase microextraction (SPME) fiber has recently been shown to have increased sensitivity for ionic compounds compared to more conventional sampler coatings such as polyacrylate and polydimethylsiloxane (PDMS). However, data for structurally diverse compounds to this (prototype) sampler coating are too limited to define its structural limitations. We determined C18/SCX fiber partitioning coefficients of nineteen cationic structures without hydrogen bonding capacity besides the charged group, stretching over a wide hydrophobicity range (including amphetamine, amitriptyline, promazine, chlorpromazine, triflupromazine, difenzoquat), and eight basic pharmaceutical and illicit drugs (pKa > 8.86) with additional hydrogen bonding moieties (MDMA, atenolol, alprenolol, metoprolol, morphine, nicotine, tramadol, verapamil). In addition, sorption data for three neutral benzodiazepines (diazepam, temazepam, and oxazepam) and the anionic NSAID diclofenac were collected to determine the efficiency to sample non-basic drugs. All tested compounds showed nonlinear isotherms above 1 mmol/L coating, and linear isotherms below 1 mmol/L. The affinity for C18/SCX-SPME for tested organic cations without H-bond capacities increased with longer alkyl chains, ranging from logarithmic fiber-water distribution coefficients (log D_{fw}) of 1.8 (benzylamine) to 5.8 (triflupromazine). Amines smaller than benzylamine may thus have limited detection levels, while cationic surfactants with alkyl chain lengths >12 carbon atoms may sorb too strong to the C18/SCX sampler which hampers calibration of the fiber-water relationship in the linear range. The log D_{fw} for these simple cation structures closely correlates with the octanol-water partition coefficient of the neutral form ($K_{ow,N}$), and decreases with increased branching and presence of multiple aromatic rings. Oxygen moieties in organic cations decreased the affinity for C18/SCX-SPME. Log D_{fw} values of neutral benzodiazepines were an order of magnitude higher than their log $K_{ow,N}$. Results for anionic diclofenac species (log $K_{ow,N}$ 4.5, pKa 4.0, log D_{fw} 2.9) indicate that the C18/SCX fiber might also be useful for sampling of organic anions. This data supports our theory that C18-based coatings are able to sorb ionized compounds through adsorption and demonstrates the applicability of C18-based SPME in the measurement of freely dissolved concentrations of a wide range of ionizable compounds.

INTRODUCTION

Solid-phase microextraction (SPME) is a simple, passive sampling technique (*Arthur & Pawliszyn 1990*). This technique has been evolving rapidly in the last decade, with innovations in coatings or extraction phases used (*Augusto et al. 2010, Spietelun et al. 2010, Xu & Lee 2014*), changes in experimental set-ups to allow for high-throughput sampling (*Souza Silva et al. 2013*), and expanding to other fields of application including forensics (*Kabir et al. 2013*), biomedical analysis (*Kataoka & Saito 2011*) and *in vivo* sampling (*Musteata 2013*).

One of the recent advances in SPME is the use of so-called "mixed-mode" coatings. These coatings employ a mixture of two extraction mechanisms, thereby increasing analyte coverage. The C18/SCX fiber, consisting of a hydrophobic phase (C18) and strong cation exchange sites (SCX), is one of these "mixed-mode" coatings. The first publication on this fiber showed increased metabolite coverage (log K_{ow} ranging between -3 to 7) compared to other SPME coatings in an untargeted metabolomic profiling study in human plasma (*Vuckovic & Pawliszyn 2011*). The authors later showed that this SPME coating could also be used *in vivo*, when it was applied in mice (*Vuckovic et al. 2011*), pigs (*Bojko et al. 2013, Bojko et al. 2014a*) and rats (*Cudjoe et al. 2013*). The major benefit of the C18/SCX fiber is the relative high sorption affinity for hydrophilic compounds, such as amino acid analogues (*Bojko et al. 2014b*), neurotransmitters (*Cudjoe & Pawliszyn 2014*) and glucuronide drug conjugates (*Boyacı et al. 2014*). These studies show the high sensitivity of C18/SCX SPME in metabolomics.

Additionally, steps have been made to elucidate the sorption mechanism of the C18/SCX fiber. Using cationic amphetamine (*Peltenburg et al. 2013, Peltenburg et al. 2015b*) and cationic amitriptyline (*Peltenburg et al. 2015b*), a large number of variables have been identified that can influence sorption to the C18/SCX fiber. In general, the C18/SCX fiber shows increased sorption affinity for ionizable compounds compared to more conventional coatings, and over a wide pH range (*Peltenburg et al. 2013, Wang et al. 2013*). Although C18/SCX fibers are not yet commercially available, these coatings could provide useful sampling tools in clinical application, where ionized or ionizable compounds are numerous, and for *in vivo* sampling, where matrix-modifying steps to ensure a large neutral fraction are impossible or undesirable.

Although the C18/SCX coating apparently has high sensitivity for cationic drugs, current data is limited to a few compounds. The chemical applicability domain of the mixed-mode SPME as a passive sampler depends to a large extent on the range of sorption affinities; not too high to (i) deplete systems, (ii) readily saturate the sampler, and/or (iii) hamper calibration of the fiber in the linear range, and not too low to meet adequate detection limits at relevant concentration ranges. Here, we present data on the sorption of various structurally different compounds to the C18/SCX fiber. We studied the sorption of a number of ionized amines and ammonium compounds with different alkyl chain lengths, to assess the influence of amine class and hydrophobicity. Additionally, we studied a large set of basic pharmaceuticals and illicit drugs with a pKa > 8 containing additional polar moieties besides the charged group, as well as 3 neutral benzodiazepines and the acidic non-steroidal anti-inflammatory drug (NSAID) diclofenac (pKa 4.0).

MATERIALS AND METHODS

Chemicals and materials

SPME fibers with mixed-mode coating (C18/propylsulfonic acid; C18/SCX) are prototype fibers provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). They are produced in a nearly identical way as commercially available biocompatible C18-SPME fibers. The C18/SCX fibers consisted of a 3 cm piece of nitinol wire with a diameter of 202 µm of which 1.5 cm contains the SPME coating, with an average thickness of 45 µm (fiber volume 524 nL), delivered without the hypodermic needle used in the biocompatible C18-SPME fiber. Both C18 and propylsulfonic acid (2-2.5% sulfur loading) are bonded on porous HPLC column grade silica material (3 µm particles, mean pore size 100 Å, total surface area ~450 m^2g^{-1}), which is then bound to the wire with a biocompatible polymeric binder (Supelco, pers.comm.). Phosphate buffered saline (PBS; pH 7.4) consisted of 138 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl (all Merck, Darmstadt, Germany) dissolved in Milli-Q water (18.2 MΩ·cm, Millipore, Amsterdam, The Netherlands). Some compounds were tested at pH 6.3 to ensure that >99% was present as the charged species. Tests at pH 6.3 were carried out using a 10 mM phosphate buffer with 50 mg/L NaN_3 and NaCl, to a total ionic strength of 150 mM Na^+. Ammonia solution (25%) was obtained from Merck, trifluoroacetic acid was obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Methanol and acetonitrile were HPLC-grade (BioSolve, Valkenswaard, The Netherlands). A list of all test compounds including molecular structures is given in the Supporting Information, table S1.

SPME procedure

Test solutions with different concentrations of analyte were made by spiking buffer using stock solutions in methanol, ensuring methanol fractions of <1%. During SPME fiber exposure, samples were placed on a roller mixer (40 rpm). After equilibrium was reached, fibers were transferred to vials containing 120 µL desorption fluid. Equilibrium times were either determined empirically or fibers were exposed for at least 18 hours. Fibers were wiped gently using a paper tissue to remove any buffer droplets before placing them in desorption fluid. Desorption fluid for all compounds consisted of 90% acetonitrile and 10% Milli-Q water with 0.1% NH_3 (of end volume), with a resulting pH of 11. After desorption and removal of the fiber, desorption solution was acidified to pH 2-3 using 60 µL 0.1 M HCl, to approximate the pH of the mobile phase (*Peltenburg et al. 2013*). To re-use the fibers, they were pooled after use, kept in desorption fluid overnight and subsequently stored in 50/50 methanol/Milli-Q at room temperature. C18/SCX SPME fibers show excellent repeatability and reproducibility, as previously described (*Peltenburg et al. 2015b*). Fiber blanks (in triplicate) were incorporated in every experiment, using buffer solutions that had not been spiked to confirm the absence of carry-over between experiments. Since these fibers are intended for single use, they were checked regularly to monitor changes in sorption capacity after repeated use. If sorption capacity was decreased, new fibers were used for the next experiment.

For the linear alkyl amines, which were analyzed using LC-MS, fibers were desorbed using 90% acetonitrile and 10% Milli-Q water with 0.1% trifluoroacetic acid (of end volume) with a pH around 2 (*Chen et al. 2012*), as linear alkyl amines are volatile in their neutral form (pH>8.5). Aqueous samples for analysis were prepared by transferring 200 µL of the aqueous phase to 600 µL of this acidic desorption fluid. The resulting sample was mixed by repeated pipetting using the same pipette tip, to minimize loss of analyte to the tip. The acidic desorption fluid contained the tertiary amine N,N-dimethyldecylamine (T10) as internal standard to account for deviations in ionization efficiency.

The data in this paper has not been published previously, with exception of amphetamine (*Peltenburg et al. 2013*), amitriptyline (*Peltenburg et al. 2015b*), diazepam (*Peltenburg et al. 2015a*), tramadol (*Peltenburg et al. 2015a*) and C12-DEA (*Wang et al. 2013*).

HPLC and LC-MS/MS parameters

All pharmaceuticals were analyzed using HPLC with either UV or fluorescence detection. Only the linear alkyl amines were analyzed using LC-MS/MS. For all equipment and parameters used, see the Supporting Information, section S2 and S3.

Quantification and data analysis

Fiber concentrations as well as aqueous concentrations were always measured. Sorption coefficients were calculated using the aqueous concentration after exposing the fiber instead of the initial concentration. A calibration curve made from aqueous concentrations before exposure was used to calculate the remaining aqueous concentration after exposure and calculate depletion using a mass balance approach. A calibration curve in acidified desorption fluid was used to calculate the concentration in fiber desorption samples and to confirm the mass balance. Calibration curves for the linear alkyl amines were made in the previously described acidic desorption fluid, again with T10 added as internal standard. For the lowest aqueous concentrations of amitriptyline, promazine, chlorpromazine and triflupromazine, quantification was not possible as these aqueous concentrations were below the LOQ of the current HPLC method. To still establish a sorption isotherm at these concentrations, the mass balance approach was used to calculate the aqueous concentration after exposure, assuming negligible sorption to the vial surfaces (as was shown by complete mass balances obtained at all other tested concentrations).

Data was plotted and analyzed using GraphPad Prism 6 for Windows. All samples were prepared in triplicate, unless specified otherwise. Data are plotted as mean ± standard deviation in both x- (measured aqueous concentrations after fiber exposure) and y-direction (measured fiber concentrations). Fiber-water sorption coefficients (log D_{fw}) are obtained by extrapolation of log linear curve to a sorbed concentration of 1 mmol/L fiber coating (at logY=0). Although the porous coating material on the C18/SCX fibers represents a specific surface area rather than a bulk sorbent volume, a fiber coating volume of 524 nL was used as calculated according the average thickness of the fiber coating and the diameter of the nitinol wire, following refs (*Peltenburg et al. 2013, Peltenburg et al. 2015b, Wang et al. 2013*).

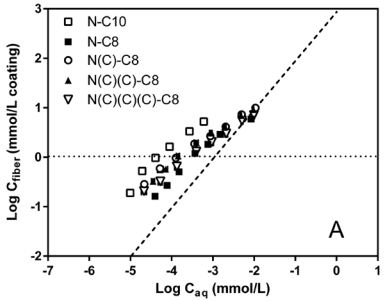
RESULTS

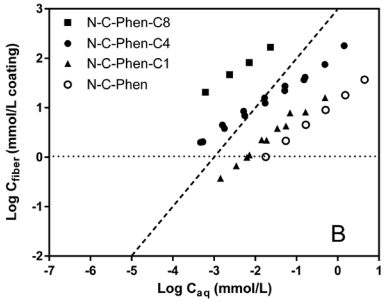
Normalizing the sorption affinity to C18/SCX at an equal chemical activity of 1 mmol/L coating

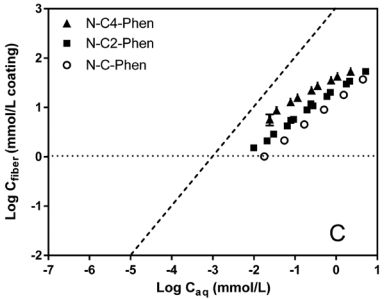
Before comparing sorption affinities between chemicals, we had to normalize the sorption affinity to a similar sorbed concentration because all currently tested compounds display nonlinear sorption isotherms over wide concentration ranges, as previously reported by us (*Peltenburg et al. 2013*) and others (*Droge & Goss 2012, Oemisch et al. 2014, Sibley & Pedersen 2008*). For all of the tested cationic and neutral compounds, C18/SCX-SPME sorption isotherm data span at least two orders of magnitude of aqueous concentrations. The anionic diclofenac was only tested at three concentrations in order to get comparable C18/SCX sorption data to measurements on the C18 fiber reported in (*Peltenburg et al. 2015b*). All tested cationic and neutral compounds showed nonlinear isotherms above sorbed fiber concentrations of 10 mmol/L coating (Figure 1). As we have discussed before (*Peltenburg et al. 2015b*), the C18-based SPME coatings are produced using highly porous silica particles for which adsorption is the main sorptive process. Apparently at loadings around 10 mmol/L, sorption sites reach critical levels where competition effects reduce the partition coefficients of both neutral and ionic compounds. Sorption eventually reaches a maximum loading (readily visualized for the dataset on the tricyclic antidepressant chlorpromazine, Figure 1). Below fiber concentrations of 1 mmol/L coating, however, the slopes of the isotherms for all compounds are all close to a value of 1 on logarithmic scale plots, suggesting that sorption is a linear process at these sorbent loadings. Since nearly all compounds were measured at, or close to, sorbed fiber concentrations of 1 mmol/L coating, we could fit the logarithmic fiber-water distribution coefficient (log D_{fw}) at 1 mmol/L coating (Tables 1-2), with exception of compound #12 (N-C-Phen-C8). Log D_{fw} was estimated using a Freundlich fit of the data below 10 mmol/L coating, thus only incorporating data in the linear concentration range. Only compound #12 (N-C-Phen-C8) was not measured below 10 mmol/L coating, so this compound was excluded from the calculations.

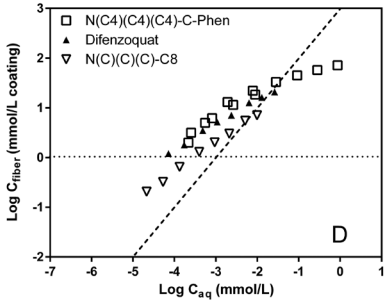
C18/SCX sorption affinity of organic cations with only C and H atoms

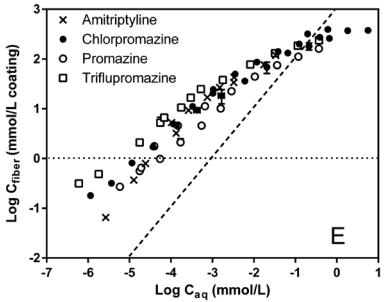
Sorption affinities to the C18/SCX fiber were determined for several series of cationic C_xH_yN structures that lack hydrogen bonding moieties besides the amine or ammonium. Compared to series of more complex pharmaceutical test

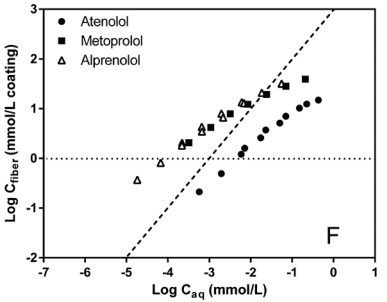

N-C10
N-C8
N(C)-C8
N(C)(C)-C8
A
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)


N-C-Phen-C8
N-C-Phen-C4
N-C-Phen-C1
N-C-Phen
B
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)


N-C4-Phen
N-C2-Phen
N-C-Phen
C
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)


N(C4)(C4)(C4)-C-Phen
Difenzoquat
N(C)(C)(C)-C8
D
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)


Amitriptyline
Chlorpromazine
Promazine
Triflupromazine
E
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)


Atenolol
Metoprolol
Alprenolol
F
Log C_fiber (mmol/L coating)
Log C_aq (mmol/L)

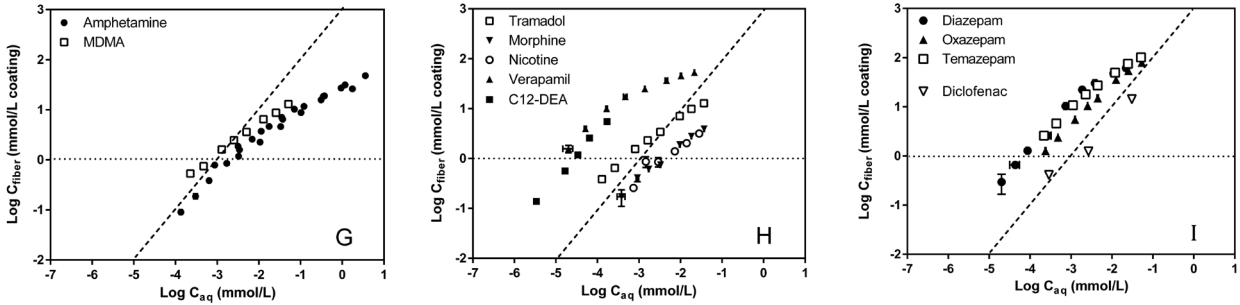


Figure 1. Sorption isotherms to the C18/SCX fiber exposed in PBS medium for 48-96h. Broken lines indicate a linear relationship between concentrations on C18/SCX fibers and dissolved concentrations with a D_{fw} of 1000 (log D_{fw} of 3). Horizontal dotted lines indicate sorbed concentrations of 1 mmol/L C18/SCX which are used to calculate log D_{fw} values. Graphs (A-D) represent organic cations without oxygen containing H-bonding functional groups. Graph (E) are tricyclic antidepressant bases (also organic cations without oxygen containing H-bonding functional groups). Graph (F) are beta-blocker bases, graph (G) amphetamine bases, graph (H) analgesic bases, the basic calcium channel blocker (verapamil), and the cationic surfactant lauryl diethanolamine (C12-DEA), graph (I) benzodiazepines and the acidic NSAID diclofenac. Data for amphetamine is taken from ref. (*Peltenburg et al. 2013*), data for amitriptyline from ref. (*Peltenburg et al. 2015b*), data for diazepam and tramadol are from (*Peltenburg et al. 2015a*), data for C12-DEA is from ref. (*Wang et al. 2013*), all with publisher permission. X- and Y-axes were kept identical to enable easy comparison.

Compound #	Amine type	Name [a])	Abbreviation [b])	Log D_{fw} [c])	Log $K_{ow,N}$ selected [d])
Same carbon chain length, different amine type					
1	NH_3^+ (1°)	1-octanamine	N - C8	3.39	3.06
2	NH_2^+ (2°)	N-methyl-1-octanamine	N (C) - C8	3.83	3.29
3	NH^+ (3°)	N,N-dimethyl-1-octanamine	N (C) (C) - C8	3.78	3.78
4	N^+ (4°)	N,N,N-trimethyl-1-octaminium	N (C) (C) (C) - C8	3.52	-
Primary amines, different carbon chains					
5	NH_3^+	1-decanamine	N - C10	4.27	4.12
6	NH_3^+	4-phenyl-1-butanamine	N - C4 - Phen	2.79	2.36
7	NH_3^+	amphetamine [e])	N - C3 - Phen	2.64	1.81
8	NH_3^+	2-phenylethanamine	N - C2 - Phen	2.26	1.46
9	NH_3^+	1-phenylmethanamine	N - C - Phen	1.76	1.09
10	NH_3^+	1-(4-methylphenyl) methanamine	N - C - Phen - C1	2.25	1.55
11	NH_3^+	1-(4-butylphenyl) methanamine	N - C - Phen - C4	3.97	3.14
12	NH_3^+	1-(4-octylphenyl) methanamine	N - C - Phen - C8	-	5.27
Quaternary amines					
13	N^+	N-benzyl-N,N-dibutyl-1-butanaminium chloride	N (C4) (C4) (C4) - C - Phen	4.28	-

Table 1. Fiber-water distribution coefficients (D_{fw}) and octanol-water partition coefficients of organic cations: simple structures containing only C, H and N.

[a]) Structures of the test chemicals are presented in Table S1 of the Supporting Information.

[b]) Abbreviation is based on the structure.

[c]) Sorption coefficients including 95% confidence interval are presented in Table S4 of the SI file.

[d]) K_{ow} values are taken from different sources (see Table S5 of the SI file).

[e]) Amphetamine is a drug, but because of its simple structure it is included in this data set. The C3 moiety contains a branched methyl group.

Compound #	Amine type	Name [a])	No. Of H-bond donor (D) and acceptor (A) [b])	Log D_{fw} [c])	Log $K_{ow,N}$ selected [d])
Tertiary amines, tricyclic compounds					
14	NH^+	Amitriptyline	-	4.51	4.92
15	NH^+	Promazine	1 A	4.56	4.55
16	NH^+	Chlorpromazine	1 A	4.84	5.41
17	NH^+	Triflupromazine	1 A	5.37	5.54
Diverse compounds					
18	NH_2^+	MDMA	2 A	3.19	2.15
19	NH^+	Tramadol	1 D, 2 A	3.31	2.51
20	NH^+	Morphine	2 D, 3 A	2.38	0.89
21	NH^+	Nicotine	1 A	2.43	1.17
22	NH^+	Verapamil	5 A	4.94	3.79
23	NH^+	C12-DEA [e])	2 D, 2 A	4.55	4.69
24	N^+	Difenzoquat [e])	-	4.29	-
Secondary amines, beta blockers					
25	NH_2^+	Atenolol	2 D, 3 A	2.34	0.16
26	NH_2^+	Metoprolol	1 D, 3 A	4.03	1.88
27	NH_2^+	Alprenolol	1 D, 2 A	4.07	3.10
Neutral compounds, benzodiazepines					
28	Neutr.	Diazepam	2 A	4.15	2.82
29	Neutr.	Temazepam	1 D, 2 A	4.02	2.19
30	Neutr.	Oxazepam	2 D, 3 A	3.67	2.24
Anionic compound					
31	COO-	Diclofenac	1 D, 2 A	2.76	4.51

Table 2. Fiber-water distribution coefficients (D_{fw}) and octanol-water partition coefficients of pharmaceutical and drugs. These organic cations containing C, H, N and in most cases H-bond donor and acceptor groups. Also, one anionic and three neutral compounds are included.

[a]) Structures of the test chemicals are presented in Table S1 of the Supporting Information.

[b]) H-bond donor and acceptor moieties were taken from (*ChemAxon, www.chemicalize.org*).

[c]) Sorption coefficients including 95% confidence interval is presented in Table S4 of the SI file.

[d]) K_{ow} values are taken from different sources (see Table S5 of the SI file).

[e]) C12-DEA is a surfactant, difenzoquat is a pesticide.

compounds, these simple cationic structures allow for a more straightforward evaluation of the influence on the C18/SCX sorption affinity of (i) amine type (comparing 1°, 2°, 3° octylamines and 4° octyltrimethylammonium), (ii) different alkyl chain lengths (alkylbenzylamines), (iii) presence of an aromatic ring, (iv) alkyl chain branching. This set of simple cations further allowed us to study the relationship between sorption affinities and simple molecular descriptors, as a framework to compare and predict the affinities of more complex organic cation structures.

Table 1 lists compounds with relatively simple structures with only C, H and N atoms. Using this small data set allows for a tentative estimation of fragment values for the contribution of simple molecular moieties, such as aliphatic carbon units, aromatic carbon units, and charged nitrogen moieties, to the fiber-water sorption coefficient. Based on multiple linear regression of data for the eleven amines, fragment values were estimated (see Table 3 and Table S6). Fragment values for the different nitrogen head groups were not significant because each fragment for the head group occurs only once in the data set. Still – using the octylamines – there is a clear trend in the influence of amine type on sorption affinities, in the order 2° ≈ 3° > 4° > 1°, with a difference of 0.4 log units between 2° and 1° octylamines (Table 1). Interestingly, 4° octyltrimethylammonium has three methyl groups attached to the nitrogen atom, but does not have the highest sorption affinity of the linear alkyl amines as would be expected based on the contribution of an additional methyl group. Quaternary ammonium compounds may show lower sorption than expected, as the charge delocalization around the nitrogen atom can be unfavorable in sorption processes. Since these simple amines were tested as >99.9% ionic species, it is unlikely that the fraction of neutral species contributed to sorption to the C18/SCX coating.

The trend in fragment values for D_{fw} follows the trend in $K_{ow,N}$ based fragments for neutral nitrogen head groups (see Table 3). Clearly, the values for the N entities for sorption to the C18/SCX are much higher than values of the same fragments for the octanol-water system because of the lack of electrostatic interactions in octanol (*Vrakas et al. 2008*).

Fragment values for aliphatic carbon and for aromatic carbon in a phenyl group were significant: 0.48 ± 0.06 for aliphatic carbon and 0.29 ± 0.07 for an aromatic carbon (Table 3). These values of 0.48 and 0.29 are very similar to fragment values for partitioning between water and octanol for neutral compounds:

Fragment	Fragment value for sorption to C18/SCX fiber	Fragment	Fragment value for partitioning to octanol (neutral compound) [d]
- $NH3^+$	-0.51 ± 0.59 [b]	- NH_2	-1.41
- $NH2^+$ (C)	-0.03 ± 0.55 [b]	- NH (C)	-0.95
- NH^+ (C)(C)	-0.08 ± 0.55 [b]	- N (C)(C)	-0.72
- N^+ (C)(C)(C)	-0.34 ± 0.55 [b]		n.a.[e]
CH_2 (aliphatic)[a]	0.48 ± 0.07 [c]	CH_2 (aliphatic)	0.49
CH (aromatic)	0.29 ± 0.09 [c]	CH (aromatic)	0.23

Table 3. Fragment values for fiber –water partitioning (K_{fw}) and octanol-water partition coefficient (see details in Table S6 of the SI).

a) No distinction is made between CH, CH2 and CH3.
b) Fragment value is not significant (see text for explanation).
c) Fragment value is significant ($p < 0.01$).
d) Derived from EpiSuite (*United States Environmental Protection Agency 2012*).
e) Fragment value for quaternary nitrogen not available.

0.49 for an aliphatic CH_2 fragment and 0.23 for an aromatic carbon atom (Table 3). The difference in these fragment values for aliphatic and aromatic carbon atoms is related to differences in molecular volume or surface area of an aliphatic hydrocarbon chain versus an aromatic hydrocarbon. The van der Waals surface area (SA) of hexane (aliphatic C6) and benzene (aromatic C6) are 178 and 110 Å^2, respectively (*Anesthetic Structure Database, asd.molfield.org*). The ratio in SA of benzene versus hexane of 0.62 is similar as the ratio in fragment values ($C_{aliphatic}$ / $C_{aromatic}$) for D_{fw} of 0.60. In addition to the influence of surface area, the hydrogen bond accepting character of an aromatic ring may also have a slight influence on sorption of aromatic compounds.

The D_{fw} of tributylbenzylammonium (compound #13) is lower than predicted via these fragments. The reason is likely the extensive charge delocalization on all branches on the amine (by one phenyl and three C4 chains) and the more bulky structure of this molecule. It also shows considerably lower affinity to C18/SCX as the simple aromatic amines. Amphetamine only has 1 branched methyl unit, and fits closely to the relationship observed for all simple aromatic amines.

C18/SCX sorption affinity of pharmaceuticals and drugs with additional hydrogen bonding moieties

To study the sorption of more polar cations to the C18/SCX fiber, we used a set of 14 different pharmaceutical and illicit drugs. Most of them are >98% cationic at test pH, with the exception of diazepam, oxazepam, temazepam (all neutral), and diclofenac (>99% anionic). Table 2 lists all log D_{fw} values (at a C_{fiber} of 1 mmol/L coating) for these more polar compounds. We found relatively high sorption affinities for all compounds tested. For the $C_XH_YN^+$ amine amphetamine, the C18/SCX fiber was shown to have increased sorption affinity compared to polyacrylate fibers (*Peltenburg et al. 2013*), and this was also shown for a more hydrophobic cationic surfactant lauryl diethanolamine (C12-DEA) (*Wang et al. 2013*). Haftka et al. measured sorption affinity of chlorpromazine to polyacrylate fibers at pH 7 and found log D_{fw} values of 3.12 (*Haftka et al. 2013*), while the C18/SCX fiber displays a 50-fold higher sorption affinity for chlorpromazine (log D_{fw} = 4.84 at pH 6.3, see table 2).

The following discussion of the sorption data to the C18/SCX fiber and the effects of chemical structure on sorption is based on a comparison with octanol-water partition coefficients of the neutral form of the compounds (log $K_{ow,N}$). Of course log $K_{ow,N}$ is typically used to describe the hydrophobicity driven sorption behavior of neutral compounds, and typically log $K_{ow,N}$ accounts for differences due to the presence of aliphatic carbon chains and aromatic rings and many polar functional groups. Experimentally derived log $K_{ow,N}$ values are available for the neutral form of several of our compounds (Tables 1 and 2), and can be predicted with limited accuracy for the remaining set of compounds, e.g. using the EPIsuite algorithm or the ACD Labs software (see Table S5 in the SI file) . Using log $K_{ow,N}$, however, precludes the analysis of quaternary ammonium compounds, since there is no neutral form for these organic salts. Since experimental log $K_{ow,N}$ values are not available for several simple $C_xH_yN^+$ amines, ACD labs estimates are used. For the set of organic cations, log $K_{ow,N}$ is a useful descriptor of the organic cations without an oxygen containing H-bond donor/acceptor group, as shown in figure 2. The tricyclic compounds agree well with the other simple amines based on their log $K_{ow,N}$. Apparently the additional sulfur atom and chlorine or fluorine atoms do not lead to a significant increase in the sorption to the C18/SCX fiber.

When these groups of organic cations are combined they give a strong simple regression for all amines, only disregarding amines with oxygen containing H-bonding functionality:

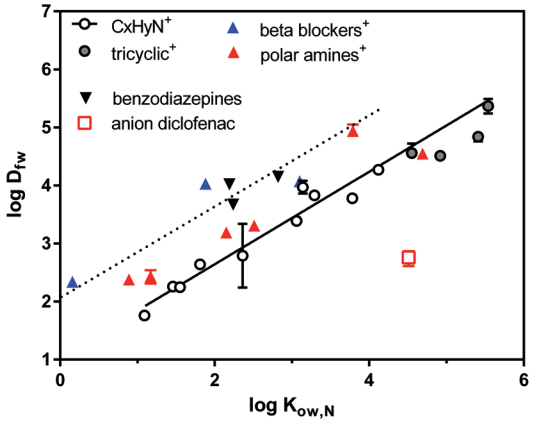


Figure 2. Relationship between octanol-water partitioning of neutral species (ACD Labs estimates for $C_xH_yN^+$ amines, experimental values for all others) and linear sorption affinity to the C18/SCX coating of the corresponding cationic species. $C_xH_yN^+$ compounds are from table 1, with exception of the quaternary amines (compounds 4 and 13, as these have no log $K_{ow,N}$) and compound 11 (as log D_{fw} could not be extrapolated accurately). The polar amines are listed in table 2, compounds 18-23. The dotted line indicates a 10x higher sorption affinity to the C18/SCX fiber compared to the log $K_{ow,N}$ relationship (line = 0.80· log $K_{ow,N}$ + 2.05).

Log $D_{fw,cation}$ (at <1 mmol/L C18/SCX) = 0.80 (±0.07) · log $K_{ow,N}$ + 1.05 (±0.19), (eq.1)

n=10, R^2=0.946, sy.x (standard deviation of the residuals, as $(SS/df)^{0.5}$) = 0.213

For three neutral benzodiazepine compounds, sorption affinity to the C18/SCX fiber does not appear to be readily predictable based on (experimentally derived) log $K_{ow,N}$ values alone. Diazepam, oxazepam and temazepam are structurally very similar but do show significant differences in sorption affinity. Compared to diazepam, temazepam has an extra OH group, which causes a decrease in sorption affinity of approximately 0.1 log units (though not significant), while experimental log $K_{ow,N}$ values differ by 0.6 log units. Oxazepam contains the same OH group but is also demethylated, further decreasing the C18/SCX sorption affinity by 0.3 log units, while experimental log $K_{ow,N}$ values do not differ between oxazepam and temazepam (Table 2).

The relationship in equation 1 illustrates again that cationic species sorb stronger than expected to the porous C18/SCX material on the mixed mode SPME fibers, taking into account that these ionized compounds also display a very high aqueous solubility. Similar conclusion can be drawn from the fragment values in Table 3. It is also interesting to note that six polar compounds are well predicted with the log $K_{ow,N}$ relationship, while three are substantial outliers that sorb much stronger to the C18/SCX fiber than predicted by the log $K_{ow,N}$ of the neutral form. The three cationic beta-blockers have a similar backbone, containing multiple polar groups, but with different substitutions on the aromatic ring: atenolol (log $K_{ow,N}$ 0.16, methylamide in para-position) and metoprolol (log $K_{ow,N}$ 1.88, methoxyethyl in para-position) sorb a factor 15 and 30, respectively, stronger to the C18/SCX material than predicted by eq. 1, while the more hydrophobic beta-blocker alprenolol (log $K_{ow,N}$ 3.10, vinyl group in ortho-position) differs only by a factor of 3. The only other polar organic cation that differs more than a factor of 5 is the large calcium channel blocker verapamil (log $K_{ow,N}$ 3.79, two aromatic rings, 4 ethers). The other polar organic cations nicotine (heterocyclic nitrogen), MDMA (methylenedioxy), tramadol (hydroxy and methoxy units), morphine (complex polycyclic diol) all sorb up to a factor 3 stronger when compared to the regression based on simple $C_xH_yN^+$ amines. The cationic surfactant C12-DEA appears to follow the sorption as predicted based on log $K_{ow,N}$, while containing 2 ethanol groups.

Other physicochemical descriptors than log $K_{ow,N}$ may be sought to derive an overall polyparameter relationship for organic cations, which includes the sorption values of atenolol, verapamil and metoprolol, to predict the linear sorption affinity to C18/SCX. For instance, (*Difilippo & Eganhouse 2010*) combined sorption affinities of hydrophobic organic compounds to polydimethylsiloxane (PDMS) coated fibers and were able to predict sorption through a polyparameter linear solvation energy relationship (LSER), using parameters related to the refractive index, polarizability and hydrogen bonding capacities in addition to the molecular volume. A similar compilation was made by (*Endo et al. 2011*) for a diverse set of neutral organic compounds, where sorption to polyacrylate coated SPME fibers could be predicted through polyparameter linear free energy relationship (PP-LFER) models. Limitation of these polyparameter predictions is that they only seem to be applicable to sorption of neutral compounds to neutral SPME coatings, and the required experimental molecular descriptors are (i) not equally relevant, and likely even different for charged chemicals, and (ii)

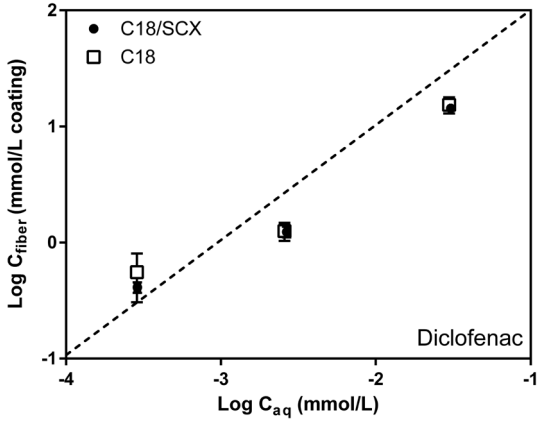


Figure 3. Comparison of sorption of diclofenac to the C18/SCX and C18 fiber. Exposure time was 72 hours. Dotted line indicates linearity.

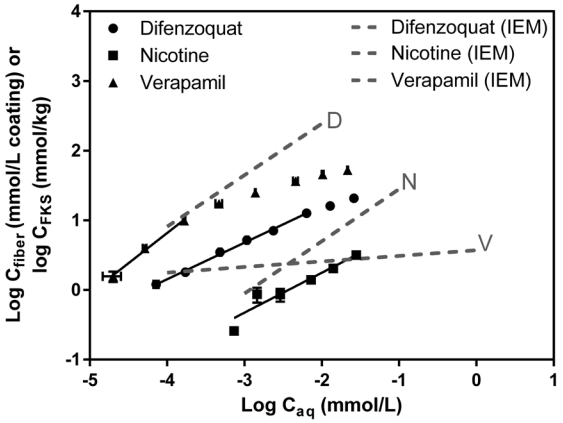


Figure 4. Comparison of sorption isotherms for difenzoquat, nicotine and verapamil to the C18/SCX fiber and ion-exchange membranes (*Oemisch et al. 2014*).

none are available for the neutral form of the tested chemicals in our study.

The predominantly anionic compounds diclofenac shows substantial sorption to the C18/SCX fiber, which is surprising as it was expected that the anionic species are repulsed from the (presumably) negatively charged C18/SCX surface. Based on its log $K_{ow,N}$, diclofenac indeed sorbs a factor 64 lower than predicted by the organic cation regression of equation 1. At pH 7.4, only 0.04% of diclofenac is present in the neutral form. Comparison of the C18/SCX fiber and C18 fiber (without strong cation exchange sites) shows equal sorption of diclofenac to both fiber types (figure 3). The strong cation exchange groups in the C18/SCX coating do not appear to inhibit sorption of anionic diclofenac. However, as sorption of diclofenac is lower than the predicted sorption based on log $K_{ow,N}$, which could indicate that both the C18 and the C18/SCX coating contain sorbent material that repulses anions. We have previously hypothesized that deprotonated free silanol groups might contribute to the sorption of cations, and counteract the sorption of anions (*Peltenburg et al. 2013, Peltenburg et al. 2015b*). However, to be able to predict sorption behavior of anionic compounds, the data set on these compounds should be extended.

Comparison of C18/SCX fibers with cation-exchange membranes

Passive sampling devices such as polar organic chemical integrative samplers (POCIS) are already used to sample ionizable chemicals in aqueous environments such as rivers and sewage treatment plants (*Jaimes-Correa et al. 2015, Vrana et al. 2016*). Although POCIS samplers can apply various polymers optimized to sorb certain types of dissolved chemicals, the polymer sorbent is typically used as an unsaturable sink, allowing for time integrated analysis of dissolved solute concentrations. The C18/SCX fiber is an equilibrium-based sampling tool that is more suitable in lab-scale partitioning studies and clinical applications. In comparison to this application of C18/SCX fibers, the use of ion-exchange membrane strips as equilibrium based sampling devices was recently evaluated for several ionized compounds (*Oemisch et al. 2014*). A cation exchange membrane (IEM) was used to study the sampler affinity in HBSS buffer (pH 7.4) for the cationic compounds nicotine, difenzoquat and verapamil. Here, we studied the sorption affinity of these compounds to the C18/SCX fiber and compared sorption affinities and Freundlich slopes (n_F) of both passive sampling devices (figure 4).
Using the IEM, good results were obtained for difenzoquat and nicotine. However, sorption of verapamil resulted in a nearly constant concentration in

Compound	This study			Oemisch et al.		
	K_F (10 µM)	n_F (0.01-1 mmol/L)	log D_{fw} at 1 mmol/L	K_F (10 µM)	n_F (1-100 µM)	log $K_{IEM/water}$ at 1 mmol/kg
difenzoquat	3.21	0.53	4.29	4.40	0.74	5.23
nicotine	2.25	0.57	2.43	2.70	0.75	2.93
verapamil	4.55	0.87	4.94	2.32	0.08	7.12

Table 4. Comparison of sorption isotherm parameters for difenzoquat, nicotine and verapamil using ion-exchange membranes (*Oemisch et al. 2014*) or C18/SCX fibers.

Distribution coefficients (log $K_{IEM/water}$ for the ion-exchange membranes and log D_{fw} for the C18/SCX fibers) are calculated at C_{aq} of 10 µM using the Freundlich equation with exponent n_F over the tested dissolved concentration range, and at a constant sorbed concentration of 1 mmol/L or mmol/kg.

the IEM at any water concentration tested, reflected by the Freundlich slope of 0.08 (*Oemisch et al. 2014*). According to the authors, the IEM was already saturated at the lowest water concentration tested. However, this is inconsistent with the total ion-exchange capacity of these membranes, which is reported at 1200 mmol/kg. Using the C18/SCX fiber, good results were obtained for all three compounds. Freundlich slopes are somewhat more nonlinear than those obtained with the IEM, with exception of verapamil (table 4). As the cation-exchange capacity of the C18/SCX fibers (~400 mmol/L coating) is a factor three lower than that of the IEM, sorption of cationic compounds to the C18/SCX fiber starts to level off at a lower fiber loading compared to the IEM.

For both passive sampling materials, molecular size and structural geometry could influence the accessibility of the ion-exchange sites. It is likely that sorption of verapamil to the IEM is limited by its large molecular size, thereby limiting the occupation of all cation-exchange sites. This compound has more predictable sorption to the C18/SCX fiber, as highlighted by the higher Freundlich coefficient. This could be the result of the highly porous nature of the C18/SCX coating, making it more accessible for larger compounds. This porosity can also have disadvantages, such as fouling of the device in protein-containing samples. A good example is fouling with bovine serum albumin (BSA). This fouling effect is larger for the IEM at low BSA concentrations, but larger for the C18/SCX fiber at high BSA concentrations (*Peltenburg et al. 2015a*).

CONCLUSION

The C18/SCX fiber has previously shown to be capable of extracting cationic compounds. Here, the data set for sorption of cationic compounds is expanded. In addition, sorption of three neutral compounds and one anionic compound is incorporated. As all compounds show sorption to the C18/SCX fiber, this strongly supports our hypothesis that ionized compounds sorb to C18-based SPME coatings through adsorptive processes. The strong cation exchange groups in the C18/SCX fiber increase sensitivity of this fiber for cationic compounds, but could also make the C18/SCX fiber more prone to competitive effects of salts, especially for polar cations with relatively low sorption affinities (e.g. log D_{fw}<2). This makes modeling sorption of ionizable compounds to the C18/SCX fiber difficult. However, there is a clear linear relationship between molecular weight and sorption affinity for alkyl amines and aromatic amines without oxygen-containing H-bonding groups. Moreover, this relationship also exists for all $C_xH_yN^+$ cations based on log $K_{ow,N}$, facilitating the expectations for the calibration feasibility of the C18/SCX fiber for related organic cation structures, e.g. cationic surfactants. More polar compounds, i.e. cations with oxygen-containing H-bonds, sorb as strong as or stronger to the C18/SCX fiber than predicted based on this log $K_{ow,N}$ relationship.

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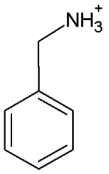
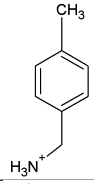
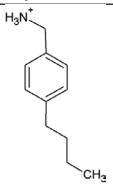
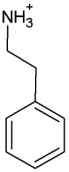
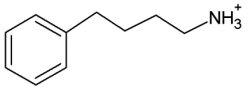
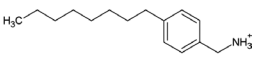
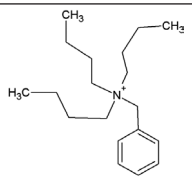
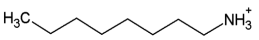
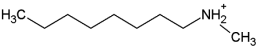
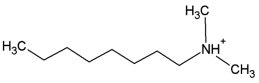
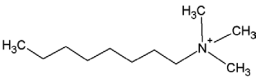
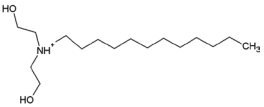
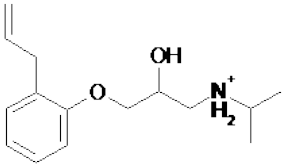
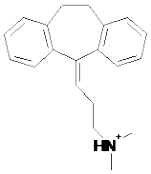
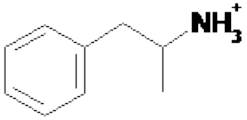
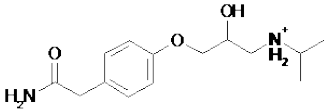
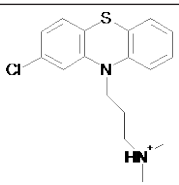
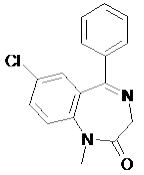
SUPPORTING INFORMATION

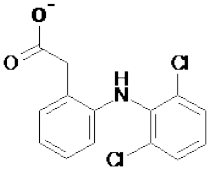
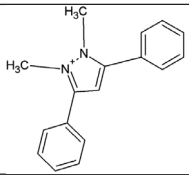
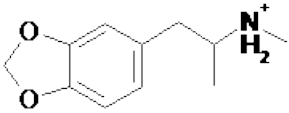
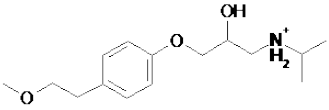
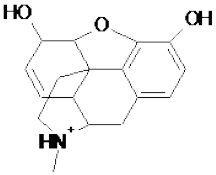
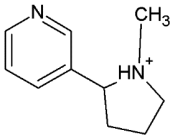
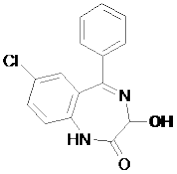
Molecular structures of all test compounds

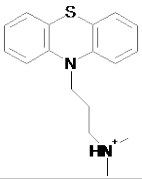
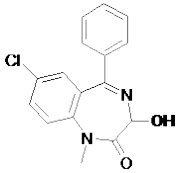
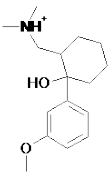
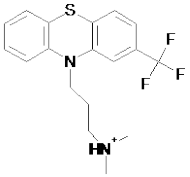
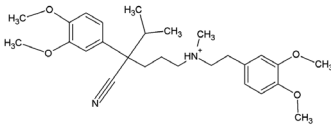
C_xH_yN compounds

Compound	Supplier	CAS number	Molecular structure
N-C-Phen 1-phenylmethanamine	Sigma-Aldrich	100-46-9	
N-C-Phen-C1 1-(4-methylphenyl)methanamine	Sigma-Aldrich	104-84-7	
N-C-Phen-C4 1-(4-butylphenyl)methanamine	Chemos	57802-79-6	
N-C2-Phen 2-phenylethanamine	Sigma-Aldrich	64-04-0	
N-C4-Phen 4-phenyl-1-butanamine	Sigma-Aldrich	13214-66-9	
N-C-Phen-C8 1-(4-octylphenyl)methanamine	Chemos	176956-02-8	

N (C4) (C4) (C4) – C-Phen N-benzyl-N,N-dibutyl-1-butanaminium chloride	Acros	54225-72-8	
N-C8t 1-octanamine	Alfa Aesar	111-86-4	
N-C10 1-decanamine	Sigma-Aldrich	2016-57-1	
N (C)-C8 N-methyl-1-octanamine	Alfa Aesar	2439-54-5	
N (C) (C)-C8 N,N-dimethyl-1-octanamine	Sigma-Aldrich	7378-99-6	
N (C) (C) (C)-C8 N,N,N-trimethyl-1-octaminium bromide	Sigma-Aldrich	2083-68-3	
C12-DEA N-dodecyl-N,N-diethanol amine	Akzo Nobel	1541-67-9	

Compound	Supplier	CAS number	Molecular structure
Alprenolol	Sigma-Aldrich	13655-52-2	
Amitriptyline	Sigma-Aldrich	50-48-6	
Amphetamine	Spruyt Hillen	300-62-9	
Atenolol	Dr. Ehrenstorfer	29122-68-7	
Chlorpromazine	Sigma-Aldrich	50-53-3	
Diazepam	Spruyt Hillen	439-14-5	

Diclofenac	Sigma-Aldrich	15307-86-5	
Difenzoquat	Sigma-Aldrich	49866-87-7	
MDMA (methylene dioxy methamphetamine)	Duchefa	42542-10-9	
Metoprolol	Dr. Ehrenstorfer	51384-51-1	
Morphine	Spruyt Hillen	57-27-2	
Nicotine	Sigma-Aldrich	54-11-5	
Oxazepam	Spruyt Hillen	604-75-1	

Promazine	Sigma-Aldrich	58-40-2	
Temazepam	Spruyt Hillen	846-50-4	
Tramadol	Sigma-Aldrich	27203-92-5	
Triflupromazine	Sigma-Aldrich	146-54-3	
Verapamil	Sigma-Aldrich	52-53-9	

Section S2
HPLC analysis parameters

The LC system used was a Prominence HPLC, consisting of two pumps, an autosampler, a column oven, a UV-detector and fluorescence detector, all from Shimadzu ('s-Hertogenbosch, The Netherlands), and a C18 column (GraceSmart RP18, ID 150 x 2.1 mm, 5 µm particle size, Grace, Breda, The Netherlands). Column oven was set at 40°C, mobile phase flow rate was 0.4 mL/min (with exception of nicotine, which was 0.2 mL/min). For all drugs, isocratic elution was achieved by using a ratio of 10 mM phosphate buffer at pH 3 and organic phase. Some linear alkyl amines were separated using a gradient. Detection was either with UV or fluorescence. For any compound that could be detected using fluorescence, UV detection was usually also employed, but the fluorescence signal was used for quantification. Used ratios of buffer and organic phase, UV wavelength and/or fluorescence excitation and emission wavelengths for each compound are summarized in the table below.

C_xH_yN compounds

Compound	Ratio buffer : organic	UV wavelength	Fluorescence ($\lambda_{ex}/\lambda_{em}$)	LOQ (nM)
N-C-Phen	97 : 3 (A)	208 nm	-	1050
N-C-Phen-C1	98 : 2 ⟶ 40 : 60 (A)	211 nm	-	400
N-C-Phen-C4	100 : 0 ⟶ 40 : 60 (A)	217 nm	-	175
N-C-Phen-C8	80 : 20 ⟶ 25 : 75 (A)	208 nm	-	1350
N-C2-Phen	96 : 4 (A)	208 nm	-	375
N-C4-Phen	91.5 : 8.5 (A)	208 nm	-	385
N(C4)(C4)(C4)-C-Phen	100 : 0 ⟶ 25 : 75 (A)	208 nm	-	200

Compound	Ratio buffer : organic	UV wavelength	Fluorescence ($\lambda_{ex}/\lambda_{em}$)	LOQ (nM)
Alprenolol	70 : 30 (A)	220 nm	230/302 nm	10
Amitriptyline	65 : 35 (A)	239 nm	-	420
Amphetamine	95 : 5 (A)	208 nm	204/280 nm	45
Atenolol	94 : 6 (A)	220 nm	230/302 nm	15
Chlorpromazine	48 : 52 (M)	255 nm	-	60
Diazepam	55 : 45 (A)	240 nm	-	175
Diclofenac	55 : 45 (A)	276 nm	-	965
Difenzoquat	70 : 30 (A)	254 nm	-	215
MDMA	90 : 10 (A)	205 nm	285/320 nm	175
Metoprolol	85 : 15 (A)	220 nm	230/302 nm	10
Morphine	95 : 5 (A)	NA	235/345 nm	1085
Nicotine	95 : 5 (A)	260 nm	-	120
Oxazepam	55 : 45 (A)	240 nm	-	65
Promazine	48 : 52 (M)	251 nm	-	50
Temazepam	55 : 45 (A)	240 nm	-	260
Tramadol	85 : 15 (A)	205 nm	200/300 nm	100
Triflupromazine	43 : 57 (M)	256 nm	-	50
Verapamil	70 : 30 (A)	210 nm	280/315 nm	10

Mobile phase composition and detection wavelengths for all study compounds. Organic phase is acetonitrile (A) or methanol (M).

Section S3
LC-MS parameters

The compounds without UV absorption, i.e. the linear alkyl amines, were analyzed using LC-MS/MS. A Perkin-Elmer liquid chromatography system (Norwalk, CT, USA) was coupled to a triple quadrupole/linear ion trap mass spectrometer (MDS Sciex API3000 LC-MS/MS System, Applied Biosystems, Foster City, CA, USA). The interface was a Turbo Ion spray source set in positive ionization mode at 4500V and operated at 400°C. Separation occurred through a Kinetex 2.6 µm XB-C18 column (50 × 2.1 mm, 100Å) with a UPLC C18

guard column. Mobile phase consisted of Milli-Q water and methanol both containing 0.1% formic acid, and each compound was run using a gradient. Run started at 5% methanol for 3.5 minutes, then increasing to 95% methanol in 1 minute. 95% methanol was maintained until 5.8 minutes, then immediately returned to 5% methanol until the end of the run (6.8 minutes). N(C)(C)-C10 is the internal standard used in the LC-MS analysis for each of the linear alkyl amines. A solvent switch was set to waste from 0 to 3.9 minutes, to MS from 4.0 until 6.4 minutes, and then to waste until end of the run. Acquisition was achieved using Analyst 1.4.2 (MDS Sciex Applied Biosystems) in selected reaction monitoring mode with fragmentation at specific m/z values.

Compound	DP (V)	FP (V)	CV (V)	M1 m/z	M3 m/z	LOQ (nM)
N-C8	70	200	16	130.1	70.9	<5
N-C10	35	200	18	158.2	71.1	<1
N(C)-C8	60	250	19	144.1	71.0	<1
N(C)(C)-C8	80	200	30	158.4	46.0	<5
N(C)(C)(C)-C8	43	292	34	172.3	60.0	<1
N(C)(C)-C10 (IS)	70	350	33	185.9	57.1	-

DP: declustering potential
FP: focusing potential
CV: collision voltage

Analysis details of C12-DEA are provided in: Wang F, Chen Y, Hermens JLM, Droge STJ, *Evaluation of passive samplers with neutral or ion-exchange polymer coatings to determine freely dissolved concentrations of the basic surfactant lauryl diethanolamine: Measurements of acid dissociation constant and organic carbon–water sorption coefficient.* Journal of Chromatography A (**2013**): 1315; 8-15.

Sorption coefficients of all test compounds including confidence interval

Compound	Log D_{fw} (95% CI)	N
1-octanamine	3.39 (3.33-3.45)	21
N-methyl-1-octanamine	3.83 (3.79-3.87)	24
N,N-dimethyl-1-octanamine	3.78 (3.73-3.84)	24
N,N,N-trimethyl-1-octaminium bromide	3.52 (3.49-3.55)	24
1-decanamine	4.27 (4.21-4.33)	17
4-phenyl-1-butanamine	2.79 (2.24-3.34)	8
Amphetamine	2.64 (2.59-2.69)	43
2-phenylethanamine	2.26 (2.20-2.31)	19
1-phenylmethanamine	1.76 (1.74-1.78)	12
1-(4-methylphenyl)methanamine	2.25 (2.20-2.30)	26
1-(4-butylphenyl)methanamine	3.97 (3.86-4.08)	18
1-(4-octylphenyl)methanamine	-	-
Amitriptyline	4.51 (4.45-4.56)	18
Promazine	4.56 (4.51-4.72)	17
Chlorpromazine	4.84 (4.76-4.91)	11
Triflupromazine	5.37 (5.24-5.49)	10
N-benzyl-N,N-dibutyl-1-butanaminium chloride	4.28 (4.11-4.45)	15
Difenzoquat	4.29 (4.25-4.34)	18
MDMA	3.19 (3.16-3.23)	21
Atenolol	2.34 (2.30-2.38)	18
Metoprolol	4.03 (3.94-4.13)	6
Alprenolol	4.07 (4.02-4.13)	16
Tramadol	3.31 (3.28-3.34)	21
Morphine	2.38 (2.32-2.43)	21
Nicotine	2.43 (2.31-2.54)	18
Verapamil	4.94 (4.84-5.05)	9
N-dodecyl-N,N-diethanol amine	4.55 (4.45-4.62)	6
Diazepam	4.15 (4.11-4.20)	27
Temazepam	4.02 (3.98-4.05)	24
Oxazepam	3.67 (3.65-3.70)	24
Diclofenac	2.76 (2.61-2.87)	6

Table S4. Sorption for 1-(4-octylphenyl)methanamine was well above 10 mmol/L and could not be extrapolated to a sorption affinity at 1 mmol/L coating.

Octanol-water partition coefficients

	Log K_{ow}		
Compound	**Exp.**	**EPIsuite**	**ACD**
1-octanamine	2.90	2.80	3.06
N-methyl-1-octanamine	-	3.27	3.29
N,N-dimethyl-1-octanamine	-	3.48	3.78
N,N,N-trimethyl-1-octaminium bromide	-	-	-
1-decanamine	-	3.78	4.12
4-phenyl-1-butanamine	2.40	2.54	2.36
Amphetamine	1.76	1.76	1.81
2-phenylethanamine	1.41	1.34	1.46
1-phenylmethanamine	1.09	1.07	1.09
1-(4-methylphenyl)methanamine	1.46	1.62	1.55
1-(4-butylphenyl)methanamine	-	3.09	3.14
1-(4-octylphenyl)methanamine	-	5.05	5.27
Amitriptyline	4.92	4.95	4.92
Promazine	4.55	4.56	4.63
Chlorpromazine	5.41	4.32	5.20
Triflupromazine	5.54	5.52	5.70
N-benzyl-N,N-dibutyl-1-butanaminium chloride	-	-	-
Difenzoquat	-	-	-
MDMA	2.15	2.28	1.81
Atenolol	0.16	-0.03	0.10
Metoprolol	1.88	1.69	1.79
Alprenolol	3.10	2.81	2.88
Tramadol	2.51	3.01	2.51
Morphine	0.89	0.72	0.43
Nicotine	1.17	1.00	0.72
Verapamil	3.79	4.80	3.90
N-dodecyl-N,N-diethanol amine	-	4.11	4.69
Diazepam	2.82	2.70	2.91
Temazepam	2.19	2.15	2.15
Oxazepam	2.24	2.32	2.31
Diclofenac	4.51	4.02	4.06

Table S5. All K_{ow} values were obtained from Chemspider (www.chemspider.com). For all pharmaceuticals, experimental values were used. In general, predicted values from ACD labs were closer to experimental values than predicted values from EPIsuite. Therefore,

predicted values from ACD labs were used for all other compounds. No log K_{ow} values are available for the three quaternary compounds (N,N,N-trimethyl-1-octaminium bromide, N-benzyl-N,N-dibutyl-1-butanaminium chloride, difenzoquat).

Fragment value calculations

Abbreviation	log Kf	N	NC	NCC	NCCC	C(al)	C(ar)
N - C8	3.39	1	0	0	0	8	0
N (C) - C8	3.83	0	1	0	0	8	0
N (C) (C) - C8	3.78	0	0	1	0	8	0
N (C) (C) (C) - C8	3.52	0	0	0	1	8	0
N - C10	4.27	1	0	0	0	10	0
N - C4 - Phen	2.79	1	0	0	0	4	6
N - C - Phen - C1	2.64	1	0	0	0	3	6
N - C2 - Phen	2.26	1	0	0	0	2	6
N - C - Phen	1.76	1	0	0	0	1	6
N - C - Phen - C1	2.25	1	0	0	0	2	6
N - C - Phen - C4	3.97	1	0	0	0	5	6

Output of multiple linear regression (performed with Graphpad Prism, version 3.0).

	Coefficients	Standard Error	P-value
Intercept	0.00		
N	-0.51	0.59	0.43
NC	-0.03	0.56	0.96
NCC	-0.08	0.56	0.89
NCCC	-0.34	0.56	0.57
C(al)	0.48	0.06	0.00
C(ar)	0.29	0.07	0.01

Coefficients are the fragment values.

Table S6. List of chemicals for calculation of fragment values, sorption coefficients to fiber and number of fragments present.

SENSITIVE DETERMINATION OF PLASMA PROTEIN BINDING OF CATIONIC DRUGS USING MIXED-MODE SOLID-PHASE MICROEXTRACTION

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ABSTRACT

Freely dissolved concentrations are considered to be the most relevant concentration in pharmacology and toxicology, as they represent the active concentration available for interaction with its surroundings. Here, a solid-phase microextraction (SPME) coating that combines octadecyl and propylsulfonic acid groups as strong cation exchange sites, known as C18/SCX or "mixed-mode" (SPME), is used to measure freely dissolved concentrations of amitriptyline, amphetamine, diazepam and tramadol to different binding matrices, including bovine serum albumin (BSA), human serum albumin (HSA), human plasma and human whole blood. A potential confounding factor in binding studies is that proteins may sorb to the fiber coating leading to incorrect measurement of protein sorption or changes in uptake kinetics to the fiber coating. Sorption of bovine serum albumin (BSA) was observed and quantified using a Lowry assay. BSA binds to the C18/SCX fiber in small amounts, but large changes in uptake kinetics were not observed. All experiments were performed at equilibrium. In addition, however, the effect of depletion and non-equilibrium extraction on the estimation of protein binding affinities was also studied. Binding affinities to BSA and human serum albumin (HSA) were calculated as log K_{BSA} or log K_{HSA}. These values were very similar to reported literature values. Sampling at either equilibrium or non-equilibrium resulted in similar binding affinities. Furthermore, SPME fibers were used to measure freely dissolved concentrations in undiluted human plasma and whole blood. Analysis of SPME extracts could be performed using HPLC-UV or HPLC with fluorescence detection without prior clean-up of the samples. Measured bound fractions in plasma using this SPME approach were comparable to literature reference values. Bound fractions in whole blood were always higher than in plasma, due to red blood cell partitioning. This work shows the potential of SPME as sampling tool for freely dissolved concentrations, especially for highly protein-bound compounds. Conventional SPME coatings such as polyacrylate (PA) or polydimethylsiloxane (PDMS) might be lacking sensitivity when sampling the small neutral fraction of highly protein-bound positively charged compounds, but the C18/SCX fiber is able to sorb the charged species of organic cations, thereby improving sensitivity for these types of compounds.

INTRODUCTION

In pharmacology and toxicology, it is generally accepted that the free concentration of a compound is more relevant than the total concentration, as only the free concentration is capable of diffusing through membranes and binding to receptors *(Seydel & Schaper 1981)*. However, both *in vivo* and *in vitro*, free concentration measurements are rarely used to plot dose response curves and calculate the 50% effect concentration (EC50). With highly protein-bound compounds, this can result in incorrect estimation of the effective or toxic dose, as binding of the drug is not taken into account. A recent compilation of plasma protein binding data for 222 different pharmaceuticals revealed that 50% of these compounds show more than 90% plasma protein binding *(Zhang et al. 2012)*, showing the importance of choosing a correct dose metric in quantitative *in vitro-in vivo* extrapolation *(Groothuis et al. 2015)*. Of these 222 drugs, 70% is positively or negatively charged at physiological pH. Anionic compounds have a higher affinity for albumin, the most abundant protein in the blood, while QSAR models show that the ionized fraction of basic drugs has a large negative impact on protein binding *(Ghafourian & Amin 2013)*. Measurement of the free concentration does not only provide information on the biologically relevant dose of a chemical but can also be used to determine the binding affinity of the compound to different binding phases *(Heringa & Hermens 2003)*, which is useful in drug discovery and drug development.

A number of different binding phases may contribute to the bound concentration of a compound *in vivo*. These include the plasma proteins serum albumin (human; HSA) and α_1-acid glycoprotein (AGP). As said, HSA is the most abundant protein in the blood. It contains two dominant drug binding sites, Sudlow sites I and II located in subdomains IIA and IIIA, respectively *(Ghuman et al. 2005)*. However, binding is not limited to these two sites as there are multiple other drug-binding sites present *(Ghuman et al. 2005, Zhang et al. 2012)*. AGP has been described as the plasma protein that primarily binds basic drugs *(Israili & Dayton 2001)*, although not all basic drugs show affinity for AGP *(Israili & Dayton 2001)* and also acidic and neutral drugs can have affinity for AGP *(Israili & Dayton 2001)*. Furthermore, both proteins are known to vary in concentration with age and disease state *(Viani et al. 1992)*, resulting in potentially large effects on free, and thus pharmacologically active, concentrations. In addition to plasma proteins as binding phase *in vivo*, highly lipophilic compounds may also bind to lipoproteins and cell membranes *(Zhang et al. 2012)*. However, protein binding studies are usually

performed using plasma *(Buscher et al. 2014)*, thereby not accounting for other blood components that could affect the free fraction.

Several methods are available to determine binding affinities and/or free concentrations. These include more conventional methods such as equilibrium dialysis and ultrafiltration, but also affinity chromatography and protein precipitation. Equilibrium dialysis is the most commonly used technique to study plasma protein binding in drug discovery and drug development *(Buscher et al. 2014)*. Although rapid equilibrium dialysis devices exist, the average incubation time for these devices still ranges between 2 and 7 hours *(Buscher et al. 2014)*, making it a time-consuming technique. Another pitfall of equilibrium dialysis is the possible dilution of the components in the solution due to osmotic volume shift *(Heringa & Hermens 2003)*. For unstable compounds and poor binders, equilibrium dialysis is usually replaced by ultrafiltration *(Buscher et al. 2014)*. For both techniques, a critical aspect is nonspecific binding at the membranes and other surfaces of the devices, which could significantly influence the measurements *(Buscher et al. 2014)*. Non-specific binding to plastic will occur in particular for more hydrophobic compounds but also cationic chemicals *(Palmgrén et al. 2006)*, making equilibrium dialysis unsuitable to study sorption processes.

Affinity chromatography uses a HPLC column packed with immobilized albumin attached to a silica support. Protein binding can be deduced from the retention time of the analyte through the column. HPLC-based determination of protein binding is faster than equilibrium dialysis or ultrafiltration, although highly bound compounds can still have retention times of more than 30 minutes *(Valko et al. 2003)*. Immobilized albumin appears to behave similar to protein in solution, but pharmacologically irrelevant interactions such as nonspecific binding to protein and binding to the silica support are known to contribute to the overall retention of the analyte *(Ascoli et al. 2006)*. For ionizable compounds in particular, retention is influenced by protonation as the net charge of the albumin surface increases partitioning for anionic compounds, while decreasing this for cationic compounds *(Chrysanthakopoulos et al. 2010)*. Even though albumin chromatography can be a useful tool to determine protein binding, especially for compounds with 90-99% protein binding *(Valko et al. 2003)*, it is not widely used in drug discovery and drug development *(Buscher et al. 2014)*.

A useful alternative to determine free concentrations is solid-phase microextraction (SPME). SPME was developed by Arthur & Pawliszyn *(Arthur & Pawliszyn 1990)* in the early 1990s as a simple extraction technique for volatile organic compounds. Vaes et al. *(Vaes et al. 1996)* first described SPME as tool to study free concentrations, where they aimed for negligible depletion of the free concentration to ensure no change in the equilibrium between bound and free concentration. Conventional SPME coatings such as polyacrylate (PA), polydimethylsiloxane (PDMS) and carbowax (CW) provided high extraction yields for neutral chemicals or neutral species of ionized chemicals. Many early studies therefore employed an increase in sample pH when sampling basic compounds to obtain large neutral fractions. Using this approach, free fractions and protein binding of for instance antidepressants *(Ulrich & Martens 1997)* could be determined, although studying protein binding at high pH lacks physiological relevance. Even small pH changes can influence outcomes of protein binding studies for ionizable compounds. As reported by *(Musteata et al. 2006)*, a pH shift in plasma from 7.4 to 8.4 has large effects on the degree of ionization of many cationic compounds, thereby significantly affecting sorption of the neutral species of these compounds to the SPME fiber. Moreover, for highly protein-bound cationic compounds, sampling the small neutral fraction that is freely available could lead to detection problems.

Negligible depletion SPME (nd-SPME) has been applied in several studies to study free concentrations and binding affinities to proteins, for example references *(Bojko et al. 2012, Kramer et al. 2007)*. However, instead of restricting the amount bound to the fiber to a negligible fraction of the dissolved concentration as is the case in nd-SPME, it is also possible to allow the free concentration to be temporarily depleted as long as the amount bound to the fiber is much smaller than the amount bound to the binding phase in the matrix *(Mayer et al. 2000)*. When measuring sorption coefficients based on a mass balance approach, depletion is in principle allowed. Binding of proteins to the fiber (so-called fouling) can alter analyte uptake, either due to an increase or decrease in fiber concentrations at equilibrium or through enhanced or reduced sorption kinetics *(Heringa & Hermens 2003, Kramer et al. 2007, Poon et al. 1999)*. If fouling significantly contributes to the concentration on the fiber surface, it may lead to incorrect estimates of the freely dissolved concentration.

Previous work has provided a mechanistic insight into sorption of organic cations to an SPME fiber coating consisting of hydrophobic C18 chains

and strong cation exchange groups (C18/SCX fiber) *(Peltenburg et al. 2013, Peltenburg et al. 2015)*. Amphetamine and amitriptyline, cationic drugs that are >99% charged at physiological pH, were used as model compounds to show the increased sensitivity of the C18/SCX fiber over more conventional SPME coatings *(Peltenburg et al. 2013)*. A following paper has shown how sorption to this fiber was affected by changing sorption conditions, including pH, ionic strength and composition, and temperature as well as through competition with other cationic drugs at higher concentrations *(Peltenburg et al. 2015)*. In the present study, the applicability of the C18/SCX fiber to study free concentrations of amitriptyline, amphetamine, diazepam and tramadol in the presence of different binding proteins was tested. This fiber's potential lies especially in the sorption of highly protein-bound cationic compounds at physiological pH. For cationic compounds in general, their small neutral fraction limits the use of conventional SPME coatings such as PA or PDMS without increasing the pH of the sample. For highly protein-bound cationic compounds, the amount of the small neutral fraction that is freely available for uptake into the fiber becomes so small that conventional coatings would not yield detectable fiber concentrations.

The C18/SCX SPME was applied to determine free concentrations and calculate binding affinities of three cationic and one neutral compound to protein. Specifically, the influence of protein concentration, analyte concentration, pre-equilibrium sampling and depletion on the calculation of binding affinities is studied. Also, the potential confounding effect of sorption of proteins to the fiber surface was studied. In addition, the SPME method is applied to spiked biological samples including undiluted human plasma and whole blood to test if SPME can be also be applied in complex biological samples.

MATERIALS AND METHOD

Chemicals and materials

Solid-phase microextraction fibers with mixed-mode (C18/propyl-sulfonic acid; C18/SCX) coating were prototype fibers provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). The fibers were 3 cm pieces of nitinol wire (202 µm diameter) with 1.5 cm of coating at an average thickness of 45 µm (total fiber volume 524 nL). Both C18 and propylsulfonic acid are bonded on porous HPLC column grade silica material which is then bound to the wire with a biocompatible polymeric binder (*Supelco, pers. comm.*).

Diazepam and amphetamine HCl were purchased from Spruyt Hillen, IJsselstein, The Netherlands. Amitriptyline HCl and tramadol HCl were from Sigma Aldrich (Zwijndrecht, The Netherlands). Phosphate buffered saline (PBS) consisted of 138 mM NaCl, 8 mM of Na_2HPO_4, 1.5 mM of KH_2PO_4 and 2.7 mM KCl (all Merck, Darmstadt, Germany) dissolved in Milli-Q water (18.2 MΩ·cm, Millipore, Amsterdam, The Netherlands). Ammonia solution (25%) was obtained from Merck. Methanol and acetonitrile were HPLC-grade (BioSolve, Valkenswaard, The Netherlands).

Bovine serum albumin (BSA; lyophilized, >96%) and human serum albumin (HSA; lyophilized, >96%) were purchased from Sigma Aldrich. Human whole blood, anticoagulated with citrate-phosphate-dextrose (CPD), was obtained from healthy consenting donors (Sanquin Blood Supply, Amsterdam, The Netherlands) and the study was approved by Sanquin's scientific committee. Human whole blood was centrifuged at 2000 g for 15 minutes (Allegra X-12R centrifuge, Beckman Coulter, Woerden, The Netherlands) to obtain undiluted plasma.

SPME procedure

Protein solutions were prepared by dissolving a certain amount of BSA or HSA in PBS. Most experiments are performed using albumin concentrations of 600 µM (40 g/L) which corresponds to physiological concentrations *(Peters Jr. 1995)*. For any experiment with protein, the sample was spiked with the compound and then placed on a roller mixer (Stuart SRT9) at 40 rpm for at least 30 minutes to ensure free and bound fraction are at equilibrium. Stock solutions were all in methanol and diluted with methanol to different working solutions, so that methanol content in the protein solutions or biological samples is always 1%. After spiking and equilibration, C18/SCX fibers were placed in the solution and exposed for a well-defined time interval (usually 24h) with vials on the roller mixer (40 rpm). Desorption of the fibers occurred in 120 µL of a solution with 90% acetonitrile and 10% Milli-Q water with 0.1% NH_3 (of end volume) with a pH around 11. The fibers were desorbed for at least 15 minutes, yielding a recovery of >96%, as previously reported *(Peltenburg et al. 2013)*. For experiments using protein solutions or biological samples, fibers were rinsed in Milli-Q water for a few seconds before transferring them to desorption fluid. Rinsing was shown to decrease the amount of protein sorbed to the fiber (see Supporting Information, figure S1), but it did not affect the amount of compound sorbed to the fiber (see Supporting Information, figure S2). After taking out the desorbed fibers, the desorption solutions

were acidified to pH 2-3 using 60 µL 0.1 M HCl. Fibers were used for several experiments, in between experiments they were stored in 50/50 methanol/water. C18/SCX SPME fibers show excellent repeatability and reproducibility as previously described *(Peltenburg et al. 2015)*. However, fibers were regularly checked to ensure no decrease in sorption capacity occurred. If this was the case, new fibers were used for the next experiment.

Lowry assay

To quantify the fouling effect on the C18/SCX fiber, a Lowry assay was performed. C18/SCX fibers were exposed in duplicate to 5 mL of a BSA solution. Three BSA concentrations were used, i.e. 150 µM, 300 µM and 600 µM. Different exposure times were used to assess the time-dependent effect of BSA fouling. For the Lowry assay, the fibers were individually placed in 1000 µL of Lowry C with 200 µL Milli-Q water. Lowry C consists of a 50:1 solution of Lowry A (200 mM Na_2CO_3, 100 mM NaOH and 0.7 mM Na-K-tartrate in Milli-Q) and Lowry B (2 mM $CuSO_4$ in Milli-Q). After 20 minutes, the fibers were removed and 100 µL of folin reagent was added. This was vortexed and incubated in the dark for at least 30 minutes. Following incubation, fluorescence was measured at 750 nm using an Infinite 200 microplate reader (Tecan Group, Giessen, The Netherlands). A BSA calibration curve was treated the same way, where the 200 µL Milli-Q water was replaced by 200 µL of different BSA concentrations. C18/SCX fibers exposed to PBS alone were used as a baseline measurement.

HPLC analysis

The LC system used was a Prominence HPLC, consisting of 2 pumps, an autosampler, a column oven, a UV-Vis detector and a fluorescence detector (all Shimadzu, 's-Hertogenbosch, The Netherlands). The column was a C18 column (GraceSmart RP18, ID 150 x 2.1 mm, 5 µm particle size, Grace, Breda, The Netherlands), column oven was set at 40°C. Mobile phase flow rate was always 0.4 ml/min and consisted of a 10 mM phosphate buffer at pH 3 and acetonitrile. Mobile phase was pre-mixed at a ratio (buffer : acetonitrile) of 95:5 for amphetamine or on-line at a ratio of 85:15 for tramadol, 65:35 for amitriptyline and 55:45 for diazepam. Amphetamine and tramadol were detected using fluorescence with $\lambda_{ex}/\lambda_{em}$ of 204/280 nm and 200/300 nm, respectively. Amitriptyline and diazepam were detected using UV at 239 nm and 240 nm, respectively. Limit of quantification was set at a signal to noise ratio of 10:1 and was 400 nM for amitriptyline, 45 nM for amphetamine, 170 nM for diazepam and 100 nM for tramadol.

Data analysis and calculation of binding affinities

Data was plotted and analyzed using Graph Pad Prism 6 for Windows. Uptake curves were fitted with a one-phase exponential association nonlinear regression with weighing $1/Y^2$.

Free concentrations (C_{free}) are calculated using a SPME calibration isotherm in PBS without binding phase (see Supporting Information, figure S3). The bound mass (A_{bound}) is calculated from this free concentration and the total concentration spiked, corrected using sample volume. The association constant of compound binding to albumin ($K_{albumin}$; K_{BSA} or K_{HSA}) can then be calculated:

$$K_{albumin} = A_{bound} / (A_{albumin} \cdot C_{free}) \quad (1)$$

with C_{free} in molar (M), resulting in an association constant $K_{albumin}$ with dimension M^{-1}.

$A_{albumin}$ represents the total amount of albumin added. In theory, binding affinities should be calculated with the remaining concentration of albumin after binding, $A_{albumin,free}$. Assuming a 1:1 binding, $A_{albumin,free}$ can be calculated as the total added concentration of albumin minus the bound concentration (of the ligand). However, as albumin potentially contains more binding sites per molecule for the compounds described here, the total concentration of albumin is used to calculate $K_{albumin}$. To avoid large variations in the calculation of the binding affinity, the fraction $A_{bound}/A_{albumin}$ is always kept below 0.1. This not only eliminates depletion of the ligand by binding to protein (not depletion by sorption to the fiber), but also makes sure that $A_{albumin,free}$ approximates $A_{albumin,total}$. This allows for a maximum variation in log $K_{albumin}$ of 0.05 log units, as is shown in a mathematical example in the Supporting Information, example S1.

RESULTS AND DISCUSSION

Equilibrium between free and bound concentration

The time needed to reach equilibrium between bound and free concentrations in a test system was studied using amitriptyline and diazepam. These compounds were spiked to BSA solutions (600 µM) and left for different time intervals, with vials on the roller mixer. After the designated time interval, C18/SCX fibers were introduced for 10 minutes. Figure 1 shows the fiber

concentrations of amitriptyline and diazepam after different time intervals. For both compounds, any equilibration time tested yielded very similar fiber concentrations, showing that only a short time is needed for bound and free concentrations to equilibrate. *(Zhang et al. 2010)* studied protein binding kinetics for carbamazepine and fluoxetine using temporal resolution SPME and showed that this was a pseudo-first-order process and that it took up to 30 minutes after introduction of the drug in the protein solution to reach equilibrium. Based on these data, we selected a minimum of 30 minutes to incubate the test compound with protein solutions before introducing the fiber to ensure equilibrium between bound and free concentrations had been reached.

Sorption kinetics in the presence of BSA

To study whether a binding matrix influences sorption kinetics of the compounds to the fiber, uptake curves were compared with and without added BSA (figure 2). The total spiked concentrations of the compounds are kept equal, so this results in lower fiber concentrations in the presence of BSA as there is a decrease in free concentration. For all compounds, uptake kinetics are not or only slightly affected with the addition of BSA, as calculated using the equilibration time $t_{95\%}$ (see table 1). In the literature, different effects of proteins on the uptake kinetics have been reported. Either no difference *(Holten Lützhøft et al. 2000)* or an increase in uptake kinetics (facilitated transport) *(Heringa et al. 2006, Kramer et al. 2007)* has been reported.

Facilitated transport will only occur if diffusion through the boundary layer is rate-limiting and if the amount of bound chemical is relatively high, as is the case for more hydrophobic compounds with higher octanol-water partition coefficients (K_{ow}) *(Heringa & Hermens 2003)*. Log K_{ow} values of our test compounds are listed in the Supporting Information, table S2 and this shows that these compounds are relatively hydrophilic. These log K_{ow} values are for the neutral compounds and the ionized molecule will even be more hydrophilic. Facilitated transport is less likely to occur for these hydrophilic drugs. Also, the sorption process is different from the earlier studies. Sorption to the C18/SCX fiber is not an absorptive but an adsorptive process *(Peltenburg et al. 2015)*, while the studies from e.g. *(Heringa & Hermens 2003)* or *(Kramer et al. 2007)* were performed with fiber coatings such as PA or PDMS and compounds that interact via an absorption process.

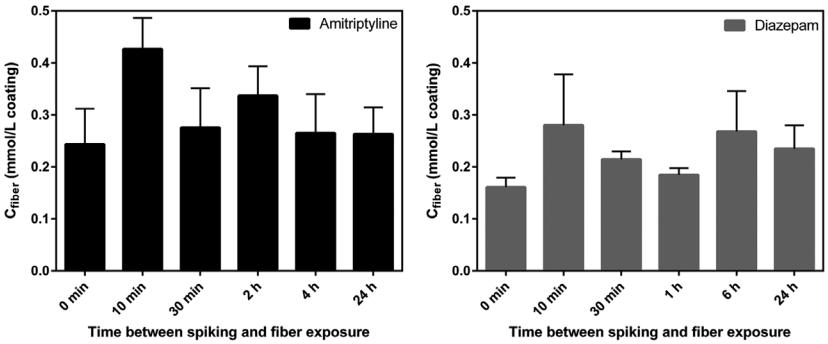

Figure 1. Fiber concentrations in the C18/SCX coating after a 10 minute exposure of C18/SCX fibers to PBS containing 600 µM BSA. Amitriptyline concentration was 3.6 µM, diazepam concentration was 5.2 µM. The time on the x-axis depicts the time interval between spiking the protein solution and introduction of the fiber. Each bar represents mean ± sd (n=3).

Compound	$t_{95\%}$, no BSA	$t_{95\%}$, 600 µM BSA
Amitriptyline	11 h (9.4 – 13)	25 h (18 – 39)
Amphetamine	0.8 h (0.7 – 1.0)	1.2 h (0.9 – 1.8)
Diazepam	10 h (8.3 – 14)	6.6 h (4.7 – 11)
Tramadol	2.5 h (2.0 – 3.2)	5.6 h (4.3 – 8.0)

Table 1. Equilibration times ($t_{95\%}$) for the four test compounds in the absence of BSA and with added 600 µM BSA. Data is calculated from data in figure 2 with 95% confidence interval.

The different sorption mechanism of the C18/SCX fiber compared to more conventional coatings, together with the porous nature of the coating, may also change the interaction between the fiber and matrix (protein) compared to that between protein and PDMS or PA coated fibers. Sorption of protein to the fiber could also alter uptake kinetics in a negative way, known as fouling.

Fouling

During some of the first experiments using BSA, a build-up of protein around the SPME fibers was noticeable after a few days of exposure. A similar visual fouling effect was described for the sampling of PCBs from human serum using PDMS fibers *(Poon et al. 1999)*. *(Heringa & Hermens 2003)* have described two potential effects of protein binding to SPME fibers. Firstly, the protein could decrease uptake of the analyte to the fiber *(Heringa & Hermens 2003)*. This would lead to slower uptake kinetics, but would only cause problems when measuring in the kinetic uptake phase. Measurements at equilibrium would not be affected, unless the protein really blocks the uptake, but that is an unrealistic scenario. Secondly, the protein could add additional analyte to the fiber, leading to higher equilibrium fiber concentrations *(Heringa & Hermens 2003)* and overestimation of the freely dissolved concentration and underestimation of the binding affinity. If both uptake to the fiber and binding to protein of the analyte are governed by hydrophobic interactions, this could be easily predicted or even modelled. However, it will become more difficult when these interactions are dictated by multiple physical-chemical properties.

To assess whether the visually observed fouling effect could be quantified, a Lowry assay was performed to measure the amount of protein bound to the fiber (figure 3). C18/SCX fibers were exposed to three different BSA concentrations for different time intervals and the amount of protein attached to each fiber was quantified. The Lowry assay showed that the observed fouling effect was indeed quantifiable and concentration dependent, but not time dependent. At 600 µM, up to 0.6 nmol of BSA sorbs to the fiber. When we assume there is only a single binding site for the analyte, this can add 0.6 nmol of analyte to the fiber corresponding to an addition of 1.1 mmol/L coating. Realistic fiber loadings of analytes with low sorption affinity and at low aqueous concentrations are of the same order of magnitude as the here observed fouling effect. So, this could potentially have a large effect on the measurement of freely dissolved concentrations. However, as saturation of protein should be avoided (see Supporting Information, example S1 and

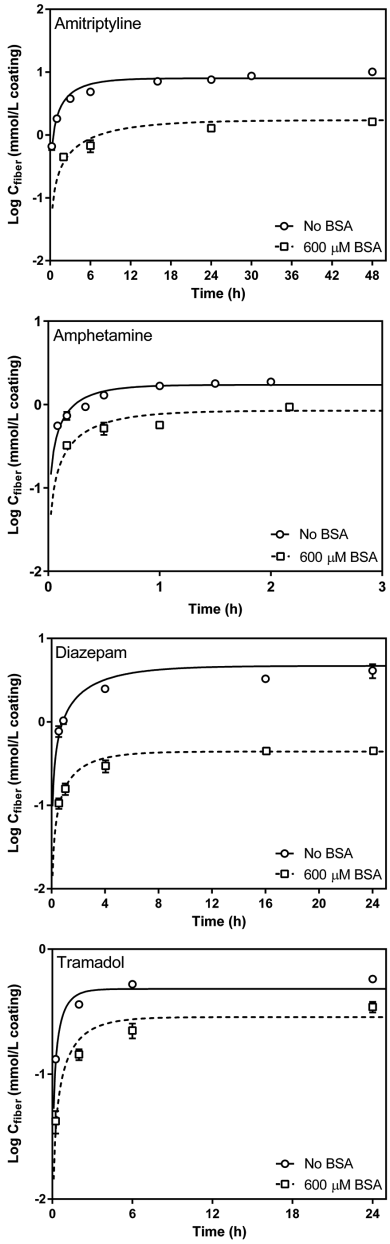

Figure 2. Uptake curves of amitriptyline, amphetamine, diazepam and tramadol using the C18/SCX fiber, comparing uptake in PBS (pH 7.4) in the presence and absence of 600 μM BSA. All uptake curves included data points for both conditions at longer time intervals, but the graphs were zoomed in to specifically show the initial uptake phase with and without protein for easy comparison. Amphetamine data without added BSA is reprinted with publisher permission from *(Peltenburg et al. 2013)*, amitriptyline data without added BSA is reprinted with publisher permission from *(Peltenburg et al. 2015)*. Please note the difference in x- and y-axis between the figures.

figure S5), analyte occupancy is always below 10%, and even below 0.1% at low aqueous concentrations. We have calculated the potential fouling effect of BSA for each compound for the results in table 2 (see Supporting Information, table S1). The largest fouling effect among the four test compounds was observed for amphetamine with 0.5% variation in log K_{BSA}. For all compounds, variation in log K_{BSA} due to fouling never exceeds 0.02 log units. This shows that the fouling effect observed for the C18/SCX fiber does not influence the calculated protein binding or binding affinity of the test compounds.

A few other studies have also attempted to quantify the amount of protein bound to passive sampling devices. *(Oomen et al. 2000)* measured binding of chyme (3.7 g/L BSA) to PDMS fibers using the Bradford assay. Binding was less than the detection limit of the assay, which translates to a potential maximum sorption coefficient of BSA for the fiber of 10 (log D_{fw} = 1). Using a more sensitive ELISA kit, sorption of HSA to PA fibers could be quantified *(Heringa et al. 2006)*, with a sorption coefficient of 2 (log D_{fw} = 0.3). Recently, *(Oemisch et al. 2014)* measured sorption of BSA to ion-exchange membranes. They reported log D_{fw} values of 1.8-2.0 at low BSA concentrations, but no sorption of BSA at concentrations above 1.5 µM. In this work, the distribution coefficient of BSA to the C18/SCX fiber is constant at all BSA concentrations tested, with a D_{fw} of 2 (log D_{fw} = 0.3). Although the C18/SCX coating contains ion-exchange sites, fouling effects for this fiber are much smaller compared to those for the ion-exchange membranes *(Oemisch et al. 2014)*. Concisely, fouling can affect measured or calculated data differently for any fiber or any analyte, so fouling effect size should always be quantified.

Binding affinities of the test compounds to BSA

The binding affinity of the test compounds to BSA was studied using three concentrations of the analyte (0.5; 5; 50 µM) and three different concentrations of BSA (150; 300; 600 µM). Every combination of analyte concentration and protein concentration was tested in triplicate. Table 2 shows the calculated binding affinities of each compound at different analyte and BSA concentrations.

At any combination, the ratio of amount of drug bound per BSA molecule was calculated to determine whether the protein starts to become saturated, which leads to a decrease in the free concentration of BSA molecules and in an underestimation of KBSA when equation 1 is applied with the total amount of albumin. At a ratio bound drug/

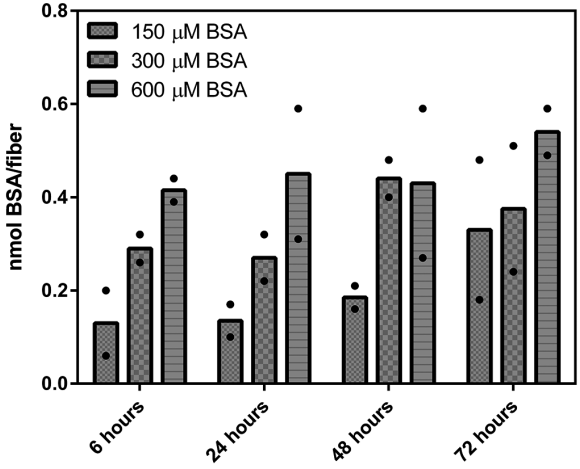

Figure 3. Sorption of BSA to C18/SCX fibers at different time intervals and BSA concentrations. Fibers were rinsed in Milli-Q water for a few seconds before transferring them to the Lowry solution. Fibers were exposed to 5 mL of the BSA concentration. Bars represent the mean of the duplicate measurement, replicates are represented by the circles.

BSA of 0.1 and higher, this underestimation becomes substantial (see Supporting Information, example S1). For each compound, this ratio is >0.1 for the combination of the lowest BSA concentration and highest analyte concentration tested (table 2; indicated by *). However, only the data for diazepam shows a clear decrease in the calculated binding affinity. For amitriptyline and tramadol, the impact of non-linearity on the calculated binding affinity is very small or not existing, while for amphetamine, there appears to be an increase in the calculated binding affinity. The absence of an effect could be explained by the presence of multiple binding sites with similar affinities. The reason why binding affinities for amphetamine increase remains unclear, although this might be related to the proximity of the fiber concentrations to the part of the calibration isotherm of amphetamine where a change in slope occurs (see Supporting Information, figure S3).

The calculated binding affinities for BSA of the different compounds correlate quite well to literature (please note that all citations for the reference values are

noted in the Supporting Information, table S3). For amitriptyline, calculated log K_{BSA} values range between 4.36 and 4.56 (excluding values where saturation has occurred) while literature values vary between 3.91, 4.06-4.34 and 4.35-4.80. For amphetamine, calculated values range between 2.74 and 3.40, with 3.03-3.15 as literature reference value. Log K_{BSA} values for diazepam have a somewhat higher variation than the other compounds, ranging between 3.80 and 4.40. Reference values range between 3.85 and 4.32. Tramadol binding affinity to BSA has a higher variation over the different concentrations tested (2.93-3.52), and also the standard deviations are higher compared to the other compounds tested. This is most likely due to the low plasma protein binding reported for tramadol (15-20% *(Baselt 2004)*). However, there are no reference values of tramadol binding affinity for BSA.

Binding affinities of the test compounds to HSA

Table 3 compares measured binding affinities of the test compounds to BSA and HSA. Here, a single analyte and albumin concentration was used. The reported binding affinities to BSA are from separate experiments than the ones reported in table 1, but the results are within the standard deviation of the measurements. BSA and HSA are 75% homologous, but clear differences are present in some clusters of amino acids, making HSA in general more hydrophobic than BSA *(Akdogan et al. 2012)*. This explains why binding affinities can strongly differ between BSA and HSA, which can especially be seen for diazepam where the difference in binding affinity between BSA and HSA is around 1 log unit. Species-dependent drug binding to albumin has been studied for diazepam *(Pistolozzi & Bertucci 2008)* and amphetamine *(Baggot et al. 1972)*. Diazepam reference values, with a binding affinity log K_{BSA} of 4.32 and log K_{HSA} of 5.41 *(Pistolozzi & Bertucci 2008)*, are very close to the values determined here (table 3).

As with reference values reported for binding to BSA, also binding affinities for HSA from literature show high variability. For amitriptyline, literature log K_{HSA} values include 3.92 and 4.27 whereas we measure 3.89±0.08 (table 3). For diazepam, literature values vary between 4.18 and 6.04, compared to a measured value of 5.22±0.06 (table 3). For tramadol, literature values and values reported here are within 0.5 log units (log K_{HSA} = 3.07-3.29 vs. 3.50±0.02). For amphetamine, no literature values are available for log K_{HSA} values.

Differences in binding affinities found in literature can stem from differences in methodologies and analytical techniques. When studying plasma protein

Compound	Measured values (log K_{BSA} ± sd)				Reference values
		150 µM	*300 µM*	*600 µM*	
Amitriptyline	0.5 µM	4.51 ± 0.09	4.56 ± 0.04	4.48 ± 0.01	3.91, 4.06-4.34, 4.35-4.80
	5 µM	4.43 ± 0.24	4.38 ± 0.17	4.36 ± 0.10	
	50 µM	4.65 ± 0.37*	4.58 ± 0.10*	4.49 ± 0.13	
		150 µM	*300 µM*	*600 µM*	
Amphetamine	0.5 µM	3.25 ± 0.31	3.16 ± 0.05	2.74 ± 0.07	3.03-3.15
	5 µM	3.40 ± 0.05	3.09 ± 0.13	3.12 ± 0.10	
	50 µM	4.03 ± 0.05*	3.72 ± 0.05*	3.39 ± 0.11	
		150 µM	*300 µM*	*600 µM*	
Diazepam	0.5 µM	4.38 ± 0.03	4.32 ± 0.02	4.19 ± 0.01	3.85, 4.32, 4.51
	5 µM	4.40 ± 0.03	4.25 ± 0.01	4.18 ± 0.02	
	50 µM	3.96 ± 0.03*	3.85 ± 0.04*	3.80 ± 0.03	
		150 µM	*300 µM*	*600 µM*	
Tramadol	0.5 µM	3.52 ± 0.33	3.04 ± 0.20	3.12 ± 0.36	N/A
	5 µM	3.46 ± 0.65	2.93 ± 0.43	3.14 ± 0.59	
	50 µM	3.65 ± 0.16*	3.18 ± 0.34	3.18 ± 0.06	

Table 2. Binding affinities to bovine serum albumin, measured using SPME and reported in literature. Measured values are in triplicate for each combination of analyte and BSA concentration and represented as log K_{BSA} ± sd. * marks measurements where the fraction bound drug/BSA was >0.1, indicating protein saturation. For tramadol, no reference values could be found (indicated by N/A). Citations for the reference values can be found in the Supporting Information, table S3.

binding using spectroscopic techniques, complete saturation of the protein is necessary, meaning that the analyte concentration should exceed the protein concentration in the sample. A nice example of this is the paper of *(Bojko et al. 2012)*, where they compared SPME with two spectroscopic methods, using 5-50 µM carbamazepine for fluorescence quenching and 50-400 µM for NMR measurements, both with 2.5 µM HSA. Their SPME technique used similar ratios between analyte and protein as used in the spectroscopic

techniques, and was shown to provide useful information on the percentage of plasma protein binding. The authors suggest that using different techniques simultaneously provides both quantitative results through SPME and structural information on complex formation through fluorescence or NMR *(Bojko et al. 2012)*. Together with the data in this paper, it is clear that SPME can be used to determine plasma protein binding in a wide range of analyte and protein concentrations, including (sub)therapeutic, toxic and lethal drug concentrations and physiological protein concentrations. Only when calculating binding affinities using SPME data, analyte to protein ratios should be calculated to avoid large variations in calculated log K_{HSA} (see Supporting Information, example S1).

SPME to measure protein binding: role of depletion and kinetic versus equilibrium sampling

In experiments with protein and SPME fibers, a compound will be distributed among a number of phases. The total amount (n_{total}) is given by:

$$n_{total}=n_{fiber}+n_{freely\ dissolved}+n_{protein} \quad (2)$$

Where n_{fiber} is the amount in the fiber coating, $n_{freely\ dissolved}$ is the amount that is freely dissolved in the aqueous phase and $n_{protein}$ is the amount bound to protein.

For measuring freely dissolved concentrations in a solution where the composition and amount of the matrix that binds the compound is not exactly known, it is important that the condition of negligible depletion of the free concentration is fulfilled. In practice, a maximum of 5% depletion aqueous ($n_{fiber} < n_{freely\ dissolved}$) is recommended *(Vaes et al. 1996)*. If this condition is not fulfilled, the uptake into the fiber may decrease the concentration bound to protein and, therefore, it will affect the freely dissolved concentration that was present before introducing the fiber. In that case, the fiber concentration is not a good representation of the freely dissolved concentration in the sample.

The depletion depends on the volume of the sample (V_s), the volume of the fiber (V_f) and the fiber –water partition coefficient (K_f) and can be calculated as *(Vaes et al. 1996)*:

$$Depletion\ (\%)=(V_f \cdot K_f)/V_s \cdot 100\% \quad (3)$$

Compound	Log K_{BSA}	Reference values BSA	Log K_{HSA}	Reference values HSA
Amitriptyline	4.41 ± 0.02	3.91, 4.06-4.34, 4.35-4.80	3.89 ± 0.08	3.92, 4.27
Amphetamine	3.19 ± 0.10	3.03-3.15	3.10 ± 0.09	N/A
Diazepam	4.16 ± 0.05	3.85, 4.32,4.51	5.22 ± 0.06	4.18, 4.42, 5.0, 5.23, 5.31 ± 0.31, 5.41, 6.04
Tramadol	3.30 ± 0.31	N/A	3.50 ± 0.02	3.07-3.29

Table 3. Binding affinities to bovine and human serum albumin. Data in each cell is log K_{BSA} ± sd or log K_{HSA} ± sd (n=3). HSA or BSA concentration was always 600 µM. Compound concentration was 5 µM, with exception of tramadol which was 0.5 µM. BSA data is from a separate experiment from that in table 2, data is in good agreement with the data presented there. For tramadol, no reference values could be found, as well as log K_{HSA} values for amphetamine (indicated by N/A). Citations for the reference values can be found in the Supporting Information, table S3.

The application of SPME in binding studies and the role of depletion has been discussed in several publications (see for example *(Bojko et al. 2012, Heringa & Hermens 2003)*). *(Musteata & Pawliszyn 2005)* even described an SPME method where significant depletion allows for the measurement of multiple data points along the binding curve, through multiple extractions in the same sample.

Depletion is acceptable when measuring freely dissolved concen-trations if:

a) the amount that is bound to protein is much larger than the amount extracted by the fiber ($n_{protein} >> n_{fiber}$).
b) the complex of a compound with the protein is labile and the amount that is taken up by the fiber is refilled from the bound form leading to a constant freely dissolved concentration during the fiber exposure.

This is also referred to as matrix SPME *(Mayer et al. 2000)*. In these studies, equilibrium is always applied because that leads to a relative simple system. In principle, depletion is also acceptable in "kinetic" SPME but then two strict conditions should be met:

c) The complex should be very labile or, in other words, desorption from the protein bound compound should be instantaneous in order to re-

establish the equilibrium between the protein-bound and freely dissolved concentration when the freely dissolved concentration is affected by uptake into the fiber coating.

d) The uptake kinetics should not be affected by the presence of the matrix.

There has been a number of studies where it was shown that proteins may facilitate the uptake into the fiber (see section 3.3). However, if specific kinetic calibration procedures are applied, it is possible to correct for these effects (see for example a study of Zhou et al. *(Zhou et al. 2008)*) and condition (d) can be ignored.

The influence of depletion and pre-equilibrium sampling was tested for diazepam. Depletion can be adjusted by using different sample volumes with equal diazepam and BSA concentrations. Figure 4 gives a graphical representation of the distribution of diazepam over the three phases present, e.g. free, bound to protein and bound to fiber. A pre-equilibrium exposure of 30 minutes was compared to equilibrium sampling. Interestingly, calculated binding affinities of diazepam to BSA show very similar log K_{BSA} values for all tested sample volumes and at both sampling times (table 4). For equilibrium sampling, depletion clearly occurs but this does not influence the calculated binding affinities. When sampling at pre-equilibrium, negligible depletion is much easier to reach, although in our experiments, the measurement of the freely dissolved concentrations and binding affinities do not seem to be influenced by the amount of depletion. This is an indication that the two conditions (c) and (d) are fulfilled.

Potential differences in uptake kinetics between samples with and without BSA should still be taken into account. Calibration of SPME fibers occurs in PBS without added binding matrix, so large differences in uptake kinetics can easily influence the calculated freely dissolved concentration and binding affinity. For the compounds tested here, either the uptake kinetics are still very similar or the difference in uptake kinetics only influences the fiber concentration to such a small extent that this does not lead to detectable differences in the calculated binding affinity. Stronger effect of protein on kinetics can be expected for more hydrophobic compounds that bind extensively to proteins *(Kramer et al. 2007)*. SPME can thus be used to measure binding affinities at equilibrium, without accounting for depletion, or at pre-equilibrium, when uptake kinetics in the absence and presence of binding matrix are similar.

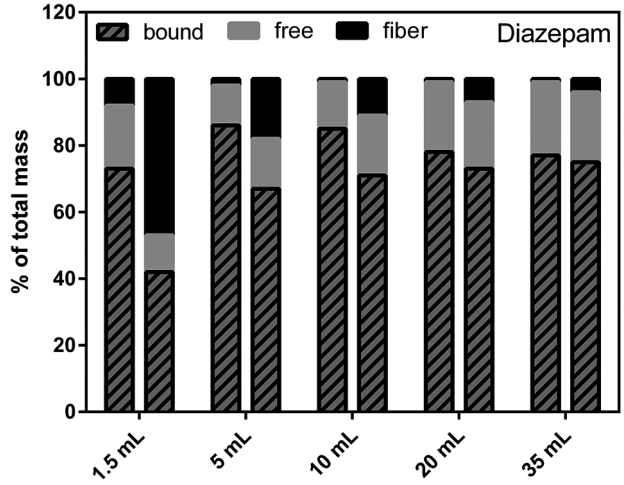

Figure 4. Graphical representation of the relative amounts of diazepam freely dissolved, bound to protein and sorbed to the fiber (average of a triplicate measurement). At each sample volume reported on the x-axis, the left bar represents the 30 minute exposure and the right bar the equilibrium exposure. Data is the average of a triplicate measurement.

Sample volume	30 min exposure		equilibrium exposure	
	Log K_{BSA} ± sd	% depletion	Log K_{BSA} ± sd	% depletion
1.5 mL	4.42 ± 0.02	8.5	4.42 ± 0.003	47
5 mL	4.67 ± 0.02	1.7	4.49 ± 0.04	19
10 mL	4.56 ± 0.10	1.1	4.44 ± 0.03	11
20 mL	4.37 ± 0.05	0.8	4.38 ± 0.004	6.5
35 mL	4.36 ± 0.04	0.4	4.38 ± 0.01	3.8

Table 4. Influence of depletion and pre-equilibrium sampling on calculated binding affinities of diazepam to BSA. Diazepam was spiked in different vial sizes at a total concentration of 5 µM. BSA concentration was 150 µM in all vials. Exposure time was either 30 minutes or at equilibrium (24 h). Free concentrations are calculated using a time-matched fiber calibration in PBS.

Application of SPME in human plasma and whole blood

To test if this specific SPME coating can also be applied in biological samples, the four test compounds were added to human whole blood or undiluted plasma. Plasma was obtained by centrifugation of whole blood, both plasma and whole blood were from the same donor. Citrate-phosphate-dextrose (CPD) was added as anticoagulant to whole blood to prevent blood clotting. The addition of CPD did not have an effect on the sorption of the test compounds to the C18/SCX fiber (see Supporting Information, figure S4).

Figure 5 shows calculated bound fractions for the different binding matrices tested compared to reference values taken from *(Baselt 2004)*. Interestingly, there are large differences for amitriptyline, amphetamine and tramadol when comparing HSA and plasma. For amphetamine and tramadol, the bound fraction in plasma is smaller than in HSA solution, while this is the other way around for amitriptyline. A few parameters could contribute to this difference. First of all, the physiological concentration of albumin in the adult human is 632 ± 53 µM *(Peters Jr. 1995)*, while for HSA alone a concentration of 600 µM was used. Furthermore, plasma contains many endogenous compounds that bind to albumin. Hydrophobic organic anions, such as long-chain fatty acids, hematin and bilirubin, have much higher binding affinities for albumin than the compounds tested here (log K_{HSA} = 7-8) *(Peters Jr. 1995)*. The presence of these compounds in plasma, but not in the HSA solution, could result in competition for binding sites, especially for low affinity compounds such as amphetamine and tramadol. Lastly, the presence of other proteins in plasma could contribute to the differences seen between plasma and HSA alone. Especially α_1-acid glycoprotein (AGP), which is attributed to be the main plasma protein for binding of organic cations *(Israili & Dayton 2001)*, could have a large contribution here. Reported literature values for amitriptyline binding affinity to AGP (log K_{AGP}) are 6.53 *(Hervé et al. 1998)* and 5.72 *(Yasgar et al. 2012)*. This means that the binding affinity of amitriptyline for AGP is 65-427 times higher compared to the binding affinity for HSA, explaining the increase in bound fraction in plasma. For diazepam, log K_{AGP} is 4.71 *(Hervé et al. 1998)*, but as this is 3-fold lower than the binding affinity of diazepam for HSA, this does not result in an increase in bound fraction.

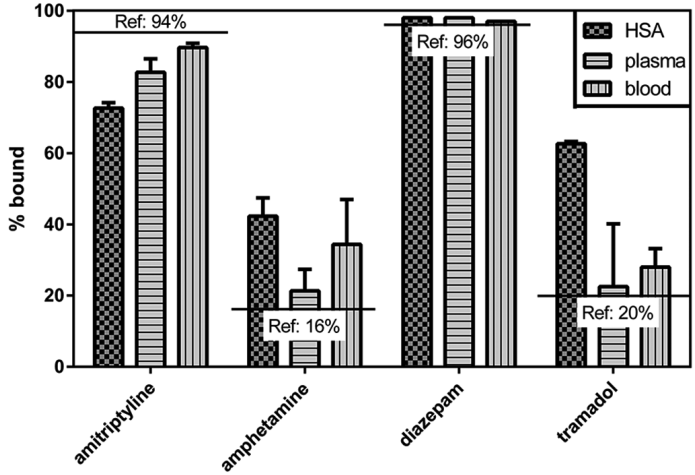

Figure 5. Plasma protein binding of amitriptyline, amphetamine, diazepam and tramadol in different binding matrices. The different binding phases include HSA, human plasma and human whole blood. HSA concentration is 600 µM. All compounds were added at a concentration of 5 µM, with exception of tramadol which was 0.5 µM. Each bar represents average and standard deviation of a triplicate measurement. % bound as found in literature *(Baselt 2004)* is indicated as reference value (Ref:).

For all compounds, bound fractions are higher in whole blood compared to plasma alone. As whole blood and plasma were obtained from the same donor, the only difference is the presence of cells in whole blood. Red blood cells make up 99% of the cells in whole blood, and red blood cell partitioning is known to have an influence on free drug concentrations *(Hinderling 1997)*. Arguably, determining drug binding in whole blood has a better predictive value than measurements in plasma or serum. Here, we show that SPME can be applied to whole blood to determine freely dissolved concentrations. Interestingly, detection of the test compounds was possible using UV or fluorescence without many interfering peaks from sorbed endogenous compounds.

CONCLUSION

The current paper discusses a specific SPME fiber coating (C18/SCX) as sampling tool to measure freely dissolved concentrations and binding affinities of compounds to different plasma proteins. Here, amitriptyline, amphetamine, diazepam and tramadol were sampled in the presence of bovine or human serum albumin (BSA or HSA). Free concentrations could be determined using SPME, spanning concentrations ranging from therapeutic to toxic and lethal concentrations *in vivo*. Calculated binding affinities of the four test compounds were similar to reported literature values. Furthermore, with the C18/SCX coating low free concentrations of highly protein-bound organic cations can be determined, whereas conventional neutral SPME coatings might not reach the same sensitivity for small free fractions of charged compounds. Application of SPME in undiluted human plasma and whole blood showed little interference from endogenous compounds. This easy application of SPME in complex matrices makes it the perfect tool to determine free concentrations, both *in vitro* and *in vivo*.

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SUPPORTING INFORMATION

Effect of rinsing on protein sorption to C18/SCX fibers

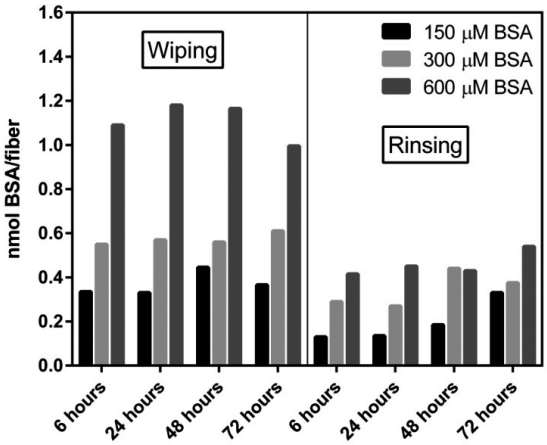

Figure S1. C18/SCX fibers were exposed in duplicate to 5 mL of different BSA concentrations in PBS. After different exposure times, fibers were removed from this solution and transferred to Lowry C solution. Fibers were either only wiped or rinsed shortly in Milli-Q water before placing them in Lowry C solution. A Lowry assay was performed as described in the Method section of the manuscript. For both conditions, sorption of BSA to the fibers was concentration-dependent but not time-dependent. Rinsing the fibers after exposure to BSA solutions clearly reduces the amount of BSA attached to the fiber up to 50%. Bars represent average values of a duplicate exposure. The data on rinsing is also displayed in figure 3 of the manuscript but included here for easy comparison between the two conditions.

Effect of rinsing on sorption of amphetamine to C18/SCX fibers

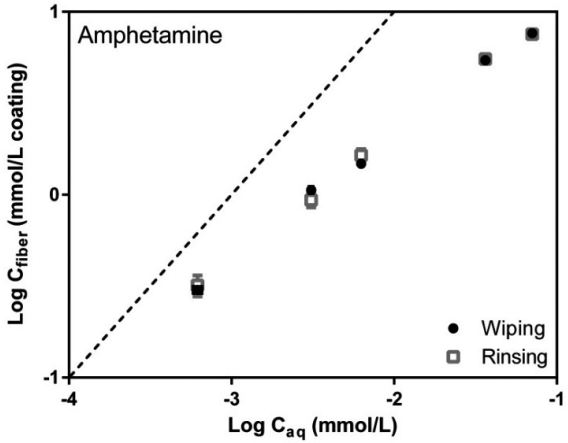

Figure S2. Rinsing the fiber after exposure to protein solutions reduces the amount of protein bound to the fiber. Here, the effect of rinsing on the sorption of the analyte was tested. C18/SCX fibers were exposed to amphetamine concentrations in PBS. Fibers were either wiped or rinsed when transferring them from exposure solution to desorption fluid. Amphetamine was chosen for this experiment as it is the least hydrophobic of the four test compounds. Therefore, it is most likely that if compounds would partition towards Milli-Q during the few seconds of rinsing, the largest effect would be seen for amphetamine. Rinsing the fiber did not affect the fiber concentration at any of the concentrations tested here.

Freundlich isotherms for SPME calibration of test compounds

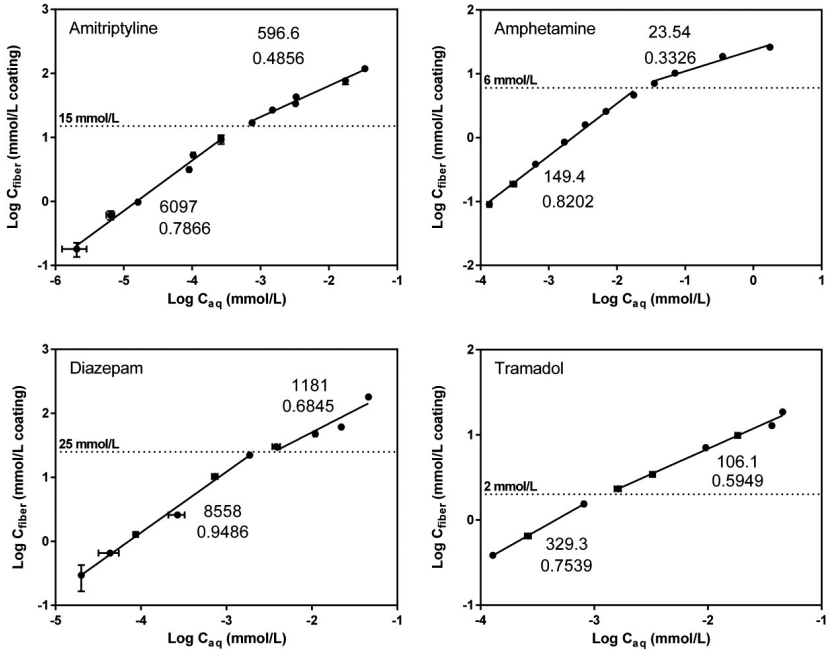

Figure S3. Sorption isotherms of the four test compounds were fitted with Freundlich isotherms. These values were used for calibration of experimental data in protein solutions. The Freundlich isotherm is as follows:

$$C_{fiber} = D_{fw} \cdot C_{aq}^{\,n} \qquad \text{or} \qquad Log\ C_{fiber} = logD_{fw} + n \cdot logC_{aq}$$

For all values, the highest value represents the D_{fw}, while the lowest value (0-1) is the exponent n. For each compound, there was a clear cut-off value where the slope of the isotherm changes. Data above this cut-off value was also fitted using the Freundlich isotherm. Amitriptyline and amphetamine data are reprinted with publisher permission from ref. 1 and 2, respectively.

Influence of CPD anticoagulant on sorption to C18/SCX fibers

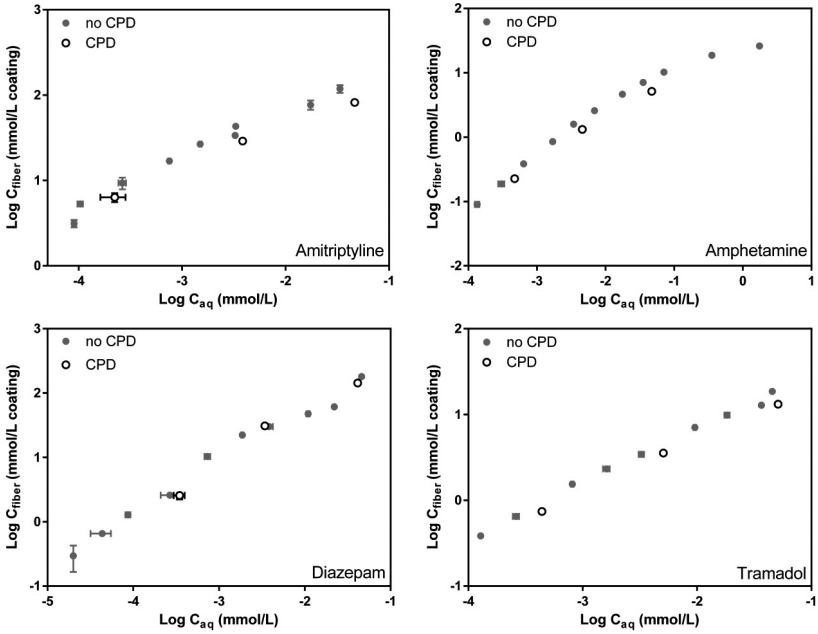

Figure S4. The influence of citrate-phosphate-dextrose (CPD), an anticoagulant used to prevent clotting of whole blood and plasma, was studied for all four test compounds. PBS (pH 7.4) was used as a reference. CPD was mixed with PBS in a ratio of 1:7 (CPD:PBS), pH of this solution was 6.4. For all compounds, sorption with or without CPD is highly comparable. Most likely, the small decrease in sorption seen for some compounds at some concentrations is mainly due to the difference in pH between the solutions.

Calculation of fouling effect size

Parameter	Not corrected	Corrected
A total (nmol)	520.8	520.8
A fiber (nmol)	3.379	3.348
C free (µM)	6.45	6.39
A bound (nmol)	313	319
A BSA (nmol)	6000	6000
Bound drug/BSA	0.05	0.05
Log K_{BSA}	3.432	3.429

Table S1. Measured and calculated data for sorption of amphetamine (50 µM) from a BSA solution (600 µM). Not corrected data is from table 1 in the manuscript. Corrected data was calculated by subtracting the maximum fouling effect (0.6 nmol) multiplied by the ratio bound drug/BSA (here 0.05 or 5%) from the measured amount of amphetamine on the fiber (A fiber). Data were bound/BSA ratio exceeds 0.1 are not included. Calculated log K_{BSA} values for this example differ 0.003 log unit. For all analytes and BSA concentrations used (table 1), this was the largest effect seen on calculated binding affinities. For all compounds, variation in log K_{BSA} was 0.02 log unit maximum (excluding data where saturation of BSA occurs). Maximum variation was 0.003 log units for amitriptyline, 0.016 log units for amphetamine, 0.002 log units for diazepam and 0.010 log units for tramadol.

Log K_{ow} and log D values for the test compounds

Compound	Log K_{ow}	Log D (pH 7.4)
Amitriptyline	4.92	2.96
Amphetamine	1.81	-0.62
Diazepam	2.91	2.92
Tramadol	2.51	0.52

Table S2. Log K_{ow} and log D values are predicted values from ACD labs, collected from the website www.chemspider.com. Please note that log K_{ow} values are for the neutral species of the compound, while amitriptyline, amphetamine and tramadol are all >99% charged at pH 7.4. Log D is the distribution coefficient between octanol and water at pH 7.4, and incorporates both ionized and unionized species.

Reference values for table 2 & 3

Compound	Ref. values BSA	Reference	Ref. values HSA	Reference
Amitriptyline	3.91	H. Glasser et al. Arch. Pharmakol. 265 (1970) 321-334.	3.92	A. Yasgar et al. PLoS ONE. 7 (2012) e45594.
	4.06-4.34	A.B. Khan et al. Colloids Surf. B Biointerfaces. 87 (2011) 447-453.	4.27	M. Chrysanthakopoulos et al. J. Chromatogr. A. 1217 (2010) 5761-5768.
	4.35-4.80	T. Banerjee et al. J. Phys. Chem. B. 110 (2006) 24147-24156.		
Amphetamine	3.03-3.15	S.P. Baker et al. J. Pharm. Pharmacol. 24 (1972) Suppl: 164P.	N/A	
Diazepam	3.85	C. Dufour et al. Biochim. Biophys. Acta. 1721 (2005) 164-173.	4.18	U. Kragh-Hansen, Mol. Pharmacol. 34 (1988) 160-171.
	4.32	M. Pistolozzi et al. Chirality. 20 (2008) 552-558.	4.42	M. Chrysanthakopoulos et al. J. Chromatogr. A. 1217 (2010) 5761-5768.
	4.51	S. Endo et al. Chem. Res. Toxicol. 24 (2011) 2293-2301.	5.0	L.A. Bagatolli et al. J. Pharm. Sci. 85 (1996) 1131-1132.
			5.23	K. Vestberg et al. Biochem. Pharmacol. 44 (1992) 1515-1521.
			5.31 ± 0.31	N.A. Kratochwil et al. Biochem. Pharmacol. 64 (2002) 1355-1374.
			5.41	M. Pistolozzi et al. Chirality. 20 (2008) 552-558.
			6.04	U. Kragh-Hansen, Biochem. J. 225 (1985) 629-638.
Tramadol	N/A		3.07-3.29	S. Tunç et al. J. Photochem. Photobiol. B. 120 (2013) 59-65.

Table S3. Reference values with literature sources for table 2 and 3 in the manuscript.

Example S1
Binding affinities based on total or free albumin concentrations

Binding affinity is calculated as follows, using total albumin concentrations:

$$K_{albumin,total} = \frac{A_{bound}}{A_{albumin,total} \cdot C_{free}} \quad \text{(eq.1)}$$

Or rewritten as function of A_{bound}:

$$A_{bound} = K_{albumin,total} \cdot A_{albumin,total} \cdot C_{free} \quad \text{(eq. 2)}$$

Or using free BSA concentrations:

$$A_{bound} = K_{albumin,free} \cdot A_{albumin,free} \cdot C_{free} \quad \text{(eq. 3)}$$

Where $K_{albumin,free}$ is the binding affinity, A_{bound} is the amount of the compound bound to albumin, $A_{albumin,total}$ is the total added amount of albumin to the system, $A_{albumin,free}$ is the amount of albumin in the system that has not bound the substrate and C_{free} is the free concentration of the compound in the system. A_{bound} and $A_{albumin}$ (free or total) should be in the same dimension (e.g. mol or nanomol etc.). With C_{free} in molar (M), $K_{albumin}$ is defined as M^{-1}.

Substituting A_{bound} in equation 1 with equation 3, then leads to the following equation:

$$K_{albumin,total} = \frac{A_{albumin,total}}{A_{albumin,free}} \cdot K_{albumin,total} \quad \text{(eq.4)}$$

Or

$$K_{albumin,total} = \frac{A_{albumin,free}}{A_{albumin,total}} \cdot K_{albumin,free} \quad \text{(eq.5)}$$

So the relationship between $K_{albumin}$ based on free or total albumin concentration can be calculated using the total and free albumin concentration and its relative change is independent from other parameters within the equation for binding affinity. This can also be extrapolated to an effect on the calculated

$K_{albumin}$ as a log value using equation 6:

$$log\ (K_{albumin,total}) = log\left(\frac{A_{albumin,free}}{A_{albumin,total}}\right) + log\ (K_{albumin,free}) \qquad \text{(eq.6)}$$

Examples

Mol ratio bound drug/albumin	albumin free/total ratio	rel. effect	effect on $K_{albumin}$ (log)
Bound drug/albumin = 0.001	$A_{albumin,free} / A_{albumin,total} = 0.999$	-0.1%	0.0004
Bound drug/albumin = 0.01	$A_{albumin,free} / A_{albumin,total} = 0.99$	-1%	0.004
Bound drug/albumin = 0.1	$A_{albumin,free} / A_{albumin,total} = 0.90$	-10%	0.05
Bound drug/albumin = 0.2	$A_{albumin,free} / A_{albumin,total} = 0.80$	-20%	0.1
Bound drug/albumin = 0.3	$A_{albumin,free} / A_{albumin,total} = 0.70$	-30%	0.16
Bound drug/albumin = 0.4	$A_{albumin,free} / A_{albumin,total} = 0.60$	-40%	0.22
Bound drug/albumin = 0.5	$A_{albumin,free} / A_{albumin,total} = 0.50$	-50%	0.3
Bound drug/albumin = 0.7	$A_{albumin,free} / A_{albumin,total} = 0.30$	-70%	0.52
Bound drug/albumin = 0.9	$A_{albumin,free} / A_{albumin,total} = 0.10$	-90%	1.0

Or presented as a graph:

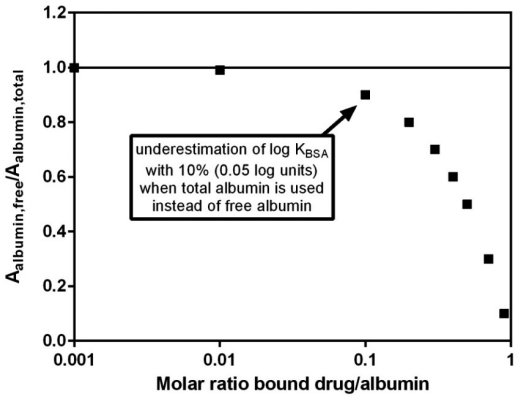

Figure S5. Ratio of K_{BSA} calculated based on total BSA concentration versus the "real" K_{BSA} based on free BSA concentration.

This graph clearly shows that at a ratio of bound drug/BSA molecule of 0.1, estimates of K_{BSA} based on total BSA concentrations starts to deviate from the real K_{BSA}.

If measurements where saturation occurs are not taken into account, i.e. all ratios of bound/albumin are ≤0.1, than the maximum variation in log $K_{albumin}$ based on total or free albumin concentration is 0.05 log units.

Note that if there are more binding sites available/present, the calculation of $A_{albumin,free}$ changes:

$$A_{albumin,free} = A_{albumin,total} - (A_{bound}/n) \quad \text{(eq. 7)}$$

Where n is the number of binding sites.

When there is more than one binding site, $A_{albumin,free}$ will therefore approximate $A_{albumin,total}$ more than with a single binding site, thus resulting in a smaller variation in binding affinities based on either total or free albumin concentrations.

1. H. Peltenburg, S.T.J. Droge, J.L.M. Hermens, I.J. Bosman, *Sorption of amitriptyline and amphetamine to mixed-mode SPME in different test conditions*, J.Chromatogr.A **2015**: 1390; 28-38.
2. H. Peltenburg, F.A. Groothuis, S.T.J. Droge, I.J. Bosman, J.L.M. Hermens, *Elucidating the sorption mechanism of "mixed-mode" SPME using the basic drug amphetamine as a model compound*, Anal.Chim.Acta **2013**: 782; 21-7.

6

DIRECT TISSUE SAMPLING OF DIAZEPAM AND AMITRIPTYLINE USING MIXED-MODE SPME FIBERS: A FEASABILITY STUDY

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Submitted to Forensic Chemistry

ABSTRACT

Recent work with solid-phase microextraction (SPME) fibers *in vivo* has shown that this technique is easily applied directly in semi-solid tissues. However, at this time data on tissue sampling is still very limited, and adequate models are lacking to study sorption from tissue. Furthermore, quantification of actual tissue concentrations remains a challenge in the application of SPME in tissue. Here, we report on the applicability of the C18/SCX fiber (containing both C18 and strong cation exchange groups) as a direct sampling tool in tissue. An agarose gel model was used as semi-solid tissue surrogate, to mimic changes in matrix tortuosity as expected in tissue. Pork muscle was used as tissue source and was loaded with the analytes of interest using 24 hour incubation in spiked phosphate-buffered saline (PBS). Diazepam and amitriptyline were used as test compounds. Diazepam is neutral and its behavior is predictable based on the octanol-water partition coefficient (K_{ow}). Amitriptyline is >99% positively charged at pH 7.4, and is likely to behave differently in both agarose gel and tissue compared to neutral compounds. The proposed SPME method yielded detectable fiber concentrations after direct sampling in agarose gel and loaded tissue, including short sampling times and different loading concentrations in tissue. These results show that the C18/SCX fiber is a sensitive tool to determine tissue concentrations of neutral and cationic compounds.

INTRODUCTION

Solid-phase microextraction (SPME) is a simple, fast passive sampling tool developed in the 1990s *(Arthur & Pawliszyn 1990)*. In recent years, *in vivo* application of SPME has gained more interest *(Kataoka & Saito 2011, Vuckovic et al. 2010)*. Most *in vivo* studies using SPME sample compounds from a liquid matrix such as blood. However, recent work has shown that SPME can also be applied in semisolid matrices such as tissue *(Ouyang et al. 2011)*. The application of SPME in tissue enables direct measurement of drug concentrations at the site of action, and allows for an empirical determination of the distribution of a compound.

The first application of SPME in tissue was in the hippocampus of mice *(Nakajima et al. 2006)*. They measured toluene after nasal inhalation by placing an SPME fiber through a cannula implanted in the brain, for 2 minutes. They observed a changing amount of toluene extracted after different inhalation concentrations and different time intervals. These measurements can be used to calculate pharmacokinetic parameters, such as elimination half-life. However, actual tissue concentrations were not calculated, as this requires correct calibration. Further studies on tissue concentrations focused on the sampling of pharmaceuticals in fish, either in a laboratory setting or after exposure to wastewater effluent *(Jahnke et al. 2009, Togunde et al. 2012a, Togunde et al. 2012b, Wang et al. 2011, Zhang et al. 2009, Zhang et al. 2010, Zhang et al. 2011, Zhang et al. 2012, Zhou et al. 2008)*.

Calculating tissue concentrations from fiber concentrations is one of the main issues regarding tissue sampling using SPME. Initially, tissue concentrations were calculated from partition coefficients generated in matrix-free solutions, such as phosphate-buffered saline (PBS) *(Zhang et al. 2009, Zhou et al. 2008)*. However, this disregards both potential fouling of the fiber and the effect of tissue density on uptake kinetics. Fouling occurs when part of the sample matrix adheres to the fiber and thereby blocks or increases the uptake of analyte *(Heringa & Hermens 2003)*. The phenomenon has been extensively described for liquid samples containing protein, see e.g. *(Peltenburg et al. 2015a)*. Recently, a mechanistic-based mathematical model was established to estimate fouling effects in liquid matrices *(Alam et al. 2015)*. In semisolid matrices such as tissue, the influence of fouling was found to be limited for the sampling of pharmaceuticals *(Zhang et al. 2010)* and polychlorinated biphenyls (PCBs) *(Jahnke & Mayer 2010)* using polydimethylsiloxane (PDMS)

fibers in fish muscle. However, fouling should be evaluated for other SPME coatings as well as other tissue types.

The different density and porosity of tissue compared to aqueous phase clearly alters the sorption behavior of a compound to the SPME fiber, as diffusion is limited or hindered by the nature of the sample matrix *(Muhr & Blanshard 1982, Zhang et al. 2011)*. This is called tortuosity, which is defined as the ratio between the length of the diffusion path and the distance between the starting and end point of the diffusing molecule. To simulate matrix tortuosity, an agarose gel model was proposed to determine rate constants of chemicals to PDMS *(Togunde et al. 2012a)*, which can then be applied in unknown tissue samples to determine tissue concentrations. Using this model, different gel concentrations can be used to mimic different tissue types, for instance, 0.8% agarose gel was found to correlate best with fish muscle *(Togunde et al. 2012a)*.

The aim of this paper is to evaluate the applicability of the C18/SCX coated SPME fiber in tissue sampling. The C18/SCX (mixed-mode) fiber coating consist of hydrophobic C18 chains and strong cation exchange (SCX) groups, made up of propylsulfonic acid. As many pharmaceuticals are bases, and therefore positively charged at physiological or environmentally relevant pH, the C18/SCX fiber was chosen as sampling tool. This fiber has been proven to be orders of magnitude more sensitive for organic cations than conventional SPME fiber coatings such as polyacrylate and PDMS *(Peltenburg et al. 2013, Peltenburg et al. 2015b)*. Furthermore, a similar mixed-mode fiber has been applied in tissue before, i.e. metabolomics profiling in lung and liver tissue of pigs *(Bojko et al. 2013)*, studying the brain metabolome of rats *(Cudjoe et al. 2013)* and sampling of methylprednisolone and its metabolites in liver and lung tissue of pigs *(Bojko et al. 2014)*. However, as of yet, no work has been done on the use of the C18/SCX fiber in direct tissue sampling as a quantitative extraction tool to determine tissue concentrations.

In this paper, the C18/SCX fiber is applied as a direct sampling tool in both agarose gel and tissue models. Previous research showed a decrease in diffusion rates of pharmaceuticals in agarose gel and tissue *(Togunde et al. 2012a)*. Here, we study uptake kinetics of diazepam and amitriptyline to the C18/SCX fiber from PBS, agarose gel and tissue. Experiments are performed under static conditions, so rate of diffusion will be the rate-limiting step in the sorption of compounds to the fiber. Diazepam and amitriptyline are used as model compounds. Diazepam is neutral at physiological pH and its behavior

in the test systems is expected to be predictable based on hydrophobicity (log K_{ow}). For amitriptyline, which is >99% cationic at pH 7.4, tissue distribution and fiber sorption are less predictable based on log K_{ow} alone. We suspect that tissue contains multiple binding sites for both chemicals, resulting in a decrease in free concentration and thus sorption to the fiber. Also, the agarose gel model might incorporate such binding groups, thereby limiting its likeness to PBS but increasing its likeness to tissue. Ultimately, we aim to show that the C18/SCX fiber is a sensitive tool that can be applied directly in tissue, both *in vitro* and *ex vivo*.

MATERIALS AND METHOD

Chemicals and materials

Diazepam and amitriptyline HCl (analytical grade) were obtained from Spruyt Hillen (IJsselstein, The Netherlands) and Sigma Aldrich (Zwijndrecht, The Netherlands), respectively. Stock solutions were prepared in methanol and stored at -20°C. Ultrapure agarose was obtained from Invitrogen (Paisley, Scotland). HPLC-grade methanol and acetonitrile were from BioSolve (Valkenswaard, The Netherlands). Ammonia solution (25%) was from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) consisted of 138 mM NaCl, 8 mM ofNa_2HPO_4, 1.5 mM of KH_2PO_4 and 2.7 mM KCl (all from Merck) dissolved in Milli-Q water (18.2 MΩ.cm, Millipore, Amsterdam, The Netherlands). PBS was set to pH 7.4 using 1 M HCl or 1 M NaOH. Prototype C18/SCX solid-phase microextraction (SPME) fibers were provided by Supelco, Sigma Aldrich (Bellefonte, PA, USA). This mixed-mode coating consist of silica particles coated with C18 and propylsulfonic acid. Fibers used here had a total length of 3 cm (nitinol wire) of which 1.5 cm is coated, at an average thickness of 45 µm.

Agarose gel preparation

To simulate the density of tissue, 1% agarose gel was used. To prepare the gels, 1 g of agarose is mixed with 100 mL of PBS in an Erlenmeyer flask. This flask is placed in a microwave and heated until just below boiling point. The flask is removed from the microwave and swirled gently to dissolve the agarose. Gels were cast in 5 mL vials which were spiked with stock solutions of diazepam or amitriptyline. The liquid agarose solution is added to the vial, and the vials are vortexed immediately to mix the agarose solution with the analyte. After vortexing, vials are left at room temperature for approximately 2 hours to allow the gel to cool and become solid. Concentrations in agarose

gel after exposure of the fiber could not be measured properly, so nominal concentrations were used for calculations and plots.

Tissue loading

Tissue was loaded with diazepam or amitriptyline using static drug exposure. Pork tissue was purchased at the local supermarket, stored at -20°C and thawed before use. The tissue was weighed and cut into pieces ranging between 1 and 2.5 g. The tissue pieces were individually exposed for 24h in 100 mL of PBS, spiked with the analyte of interest. For most experiments, a concentration of 10 mg/L was used. After 24h, the pieces were removed and blotted dry before placing the SPME fibers in the tissue.

SPME procedure

SPME fibers were conditioned before use by placing them overnight in a solution of 50:50 methanol : Milli-Q water. Fibers were exposed to agarose gel or tissue by carefully inserting the fiber in the center of the gel or tissue. After a set exposure time, usually a few hours, fibers were removed from the sample and immediately desorbed. Desorption fluid consisted of 90% acetonitrile and 10% Milli-Q water, with 0.1% NH_3 to reach a pH of 11. Desorption of the C18/SCX fiber reaches >96% recovery after 15 minutes *(Peltenburg et al. 2013)*. Prior to HPLC analysis, the desorption fluid is acidified using 0.1 M HCl (ratio 2:1 desorption fluid : HCl). A triplicate was used for each data point, i.e. three individually loaded tissue pieces with one SPME fiber exposed to each piece. All experiments incorporated blanks (e.g. fibers exposed to unspiked agarose gel or to tissue pieces placed in blank PBS for 24h).

Drug concentration in tissue

Drug concentrations in tissue after exposure of the fiber were determined to obtain distribution coefficients between tissue and the SPME fiber. Tissue samples were extracted using a liquid-liquid extraction, using acetonitrile at subzero temperatures as described by *(Yoshida & Akane 1999)*. For this method, a single smaller sample (approximately 0.5 g) of each piece of loaded tissue was taken and placed in 2 mL of Milli-Q water. The tissue is then homogenized using an Ultra Turrax (IKA, Staufen, Germany). Of this homogenate, 125 µL is transferred to an Eppendorf tube, mixed with 375 µL acetonitrile and vortexed. To precipitate all solids from the sample, it is centrifuged at 18000 rcf for 10 minutes. The sample is then placed at -20°C for at least 30 minutes. The aqueous phase and the acetonitrile will separate and the analytes of interest will be found in the upper organic layer *(Yoshida & Akane 1999)*. Phase

separation cannot be visually observed, so care should be taken to only obtain the upper organic layer. For HPLC analysis, 125 µL of the top acetonitrile layer is transferred to an HPLC vial. Recovery (and standard deviation) of diazepam and amitriptyline was 75±6% and 89±5%, respectively, based on a n=10 recovery test.

Water and fat content of tissue

Water content was determined by placing pieces of tissue in a Sanyo incubator (Osaka, Japan) at 37°C. At several time points, the tissue was weighed until a constant weight was reached. Fat content was determined using an adapted version of the Bligh & Dyer extraction. This method used approximately 0.5 g of tissue, which was mixed with 0.5 mL of Milli-Q water and homogenized using an Ultra Turrax. After adding 2 mL of methanol and 1 mL of chloroform, the sample is vortexed and placed in an ultrasonic bath for 5 minutes. This is repeated after adding another 1 mL of chloroform, and again after addition of 1 mL of Milli-Q water. The sample is then centrifuged at 2500 rpm for 15 minutes, leading to separation into three phases. The bottom phase is recovered for around 90% using a Pasteur pipette and placed in a 10 mL vial. The extraction procedure is repeated twice by adding 2 mL of chloroform. After the third extraction, the 10 mL vial contains around 6 mL of liquid which is then dried under a gentle stream of nitrogen, leaving only the fat from the extracted tissue. Due to the low amount of fat present in the tissue and the large variation between tissue suppliers, the calculated fat percentage cannot be determined with very high accuracy.

HPLC analysis

The Prominence HPLC system used consisted of two pumps with internal degasser, column oven (set at 40°C), an autosampler and a UV detector (all from Shimadzu, 's Hertogenbosch, The Netherlands). The column used was a GraceSmart 150 x 2.1 C18 column, with 5 µm particle size (Grace, Breda, The Netherlands). Flow rate was always 0.2 mL/min, mobile phase always consisted of (A) 10 mM phosphate buffered Milli-Q water (pH 3) and (B) acetonitrile. Both diazepam and amitriptyline were separated from endogenous compounds using a gradient. For diazepam, mobile phase B composition started at 40% for 2.5 minutes, then increasing to 47.5% for the next 7.5 minutes. At 10 minutes, B composition was instantly brought back to 40% and maintained there until the end of the run (12 minutes). For amitriptyline, B composition started at 35% for the first 5 minutes, was then reduced to 10% for 1.5 minutes and then returned to 35% until the end of the

run (8 minutes). UV detection of diazepam was set at 254 nm, amitriptyline at 240 nm. Limit of quantification (LOQ) was 500 µg/L for diazepam and 420 µg/L for amitriptyline.

Data analysis

Data was plotted and analyzed using Graph Pad Prism 6 for Windows. Uptake curves were fitted with a one-phase exponential association nonlinear regression with weighing $1/Y^2$. Sorption isotherms were fitted using the Freundlich equation with weighing $1/Y^2$.

RESULTS AND DISCUSSION

SPME sampling in agarose gel

Agarose gels (1%) were used to mimic diffusion in a semisolid tissue. First, the uptake of diazepam and amitriptyline from agarose gel to the C18/SCX fiber was studied (figure 1). Sorption affinity is defined as the distribution coefficient, calculated as C_{fiber} divided by the measured concentration in the sample after exposure to the fiber. Only for agarose gels, nominal concentrations were used, defined as the nominal amount spiked divided by the total volume of the hydrated agarose gel, because of difficulties extracting the analytes from the agarose matrix. No losses via evaporation or microbial degradation are expected since this was not observed in the tissue loading procedure, but it is not clear how much of the compounds is sorbed to the agarose organic complex, spatially/temporarily trapped in the agarose gel complex, or freely dissolved in the pores of the agarose gel structure. Calculated equilibrium time for uptake from agarose gel into the C18/SCX fiber coating is around 56 hours for diazepam and around 11 hours for amitriptyline (figure 1). Both for diazepam and amitriptyline, the C18/SCX sorption affinity at this exposure gel concentration does not correspond to the C18/SCX sorption affinity from PBS (figure 5), indicating a reduced free concentration due to sorption to the agarose gel phase. Agarose gel contains sorption sites for organic and inorganic compounds and these sorption sites are related to the presence of impurities *(Fatin-Rouge et al. 2003)*.

The uptake of diazepam from 0.9% agarose gel to PDMS fibers appeared to reach equilibrium after 66 hours *(Togunde et al. 2012a)*, which is comparable to the equilibrium time found here. Zhou et al. *(Zhou et al. 2008)* used 1% agarose gels to study the uptake kinetics of four cationic compounds (diltiazem, diphenhydramine, fluoxetine and norfluoxetine) to PDMS fibers. They found

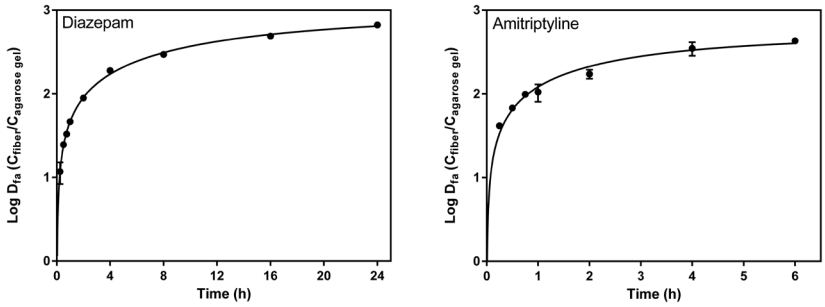

Figure 1. Uptake of diazepam and amitriptyline from 1% agarose gel to the C18/SCX fiber. Distribution coefficients (log D_{fa}) are calculated as $C_{fiber}/C_{agarose\ gel}$. Nominal concentrations were used for $C_{agarose\ gel}$, which is 1 mg/L gel for both diazepam and amitriptyline. Please note the difference in x-axis between the graphs.

equilibration times of 200 minutes (3.3 hours) for all drugs. This is three times faster than the here observed equilibration time for amitriptyline, most likely due to the much lower affinity to the (neutral) PDMS coating of these drugs which requires mass transfer of a lower amount of the compound to the PDMS and thus reaching equilibrium faster. A more detailed discussion of the equilibration times and uptake kinetics is presented in section 3.2.3.

To study the linearity of sorption from agarose gels to the C18/SCX fiber, concentration dependent sorption of the test compounds was tested (figure 2). Sorption was tested at several time points (all pre-equilibrium), and found to be linear over the entire concentration ranged tested. Freundlich slopes (n_F) range between 0.91 and 1.00 for diazepam and between 1.00 and 1.11 for amitriptyline. This linearity corresponds to the isotherm results in extraction studies from aqueous media with the same compounds (Chapter 4), which were also linear below a fiber loading of 1-10 mmol/L.

The observed difference in sorption affinity for both diazepam and amitriptyline between PBS and agarose gel could therefore indicate that agarose itself also contains groups that sorb these compounds, thereby reducing their free concentration. For instance, agarose gel has been reported to contain negatively charged groups such as pyruvate and sulfate *(Fatin-Rouge et al. 2003, Lengyel & Guttman 1999)*, which could bind cationic amitriptyline. These

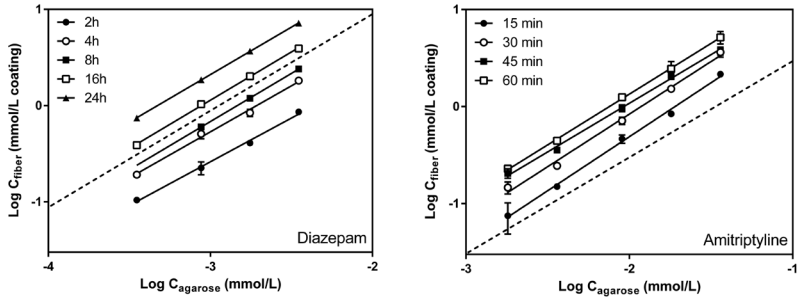

Figure 2. Concentration dependent sorption of amitriptyline and diazepam from agarose gels. Agarose gel concentrations are nominal concentrations. Dashed lines correspond with a slope of 1 (linear sorption). Please note the difference in x-axis between the graphs, and different time steps for the used symbols in both graphs. Previous work on the C18/SCX fiber found linear sorption isotherms in aqueous media below a fiber concentration of 1-10 mmol/L coating (Chapter 4).

results with agarose gels show that careful calibration of the C18/SCX SPME fiber can be used to quantitatively determine concentrations in a semisolid phase after a certain exposure period.

SPME sampling in tissue

Water and fat content of tissue

Both water and fat content of the pork muscle tissue were determined. Water content was determined by dehydrating the tissue overnight. This resulted in an average water content of 70% (figure 3). The calculation of the water content of the tissue can help to distinguish whether loading of the tissue occurs through replacement of all water content with spiked PBS, or whether there is actual uptake into the tissue itself. For the most commonly used loading concentration (10 mg/L), replacement of the water content of the tissue with spiked PBS would lead to a tissue concentration of 7 mg/kg. Average fat percentage was 3%, although this varied between 2 and 4% based on the supplier of the tissue. The contribution of storage fat and cell membranes (containing charged phospholipids) to the fat percentage is unclear, but could be important to determine sorption behavior (especially of cationic compounds).

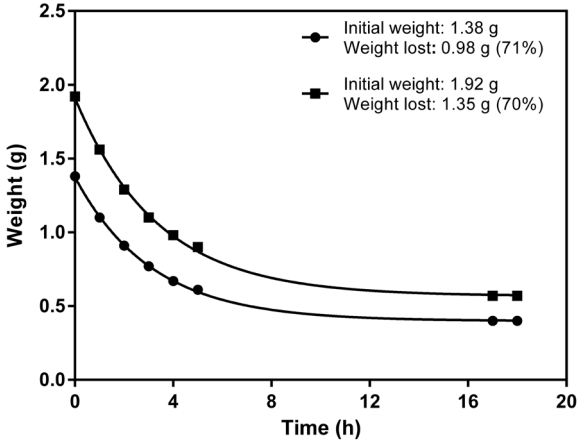

Figure 3. Weight loss by desiccation of pork muscle tissue. Water content is around 70%.

Loading muscle tissue

Contrary to the loading of agarose gels, which was simply mixed with spiked water and then solidified, pork tissue (muscle) was loaded with the test compounds by placing small pieces (1-2.5 g) in a spiked PBS solution, and the test chemicals had to permeate into the blocks of tissue. Tissue concentrations of diazepam after 4 or 24h exposure were determined by using a liquid-liquid extraction of a smaller piece of the exposed cube. Concentrations measured in the tissue are presented in figure 4A. As expected, incubation for 24h yields a higher tissue concentration than a 4h incubation. Furthermore, we tested different loading concentrations of diazepam in 24h exposure systems (figure 4B). The concentrations in tissue increase with the amount spiked in the loading medium, with the highest concentration giving the highest standard deviation. It is not yet clear to what extent the 24h loading time has equilibrated the pork tissue with the medium. However, with a log K_{ow} of 2.91 and, of more direct relevance, a liposomal affinity (LogK_{mw}) of 3.0 *(Ottiger & Wunderli-Allenspach 1997)*, a 10 mg/L solution should equilibrate to a concentration sorbed to fat of 10 g/kg fat. With 3% fat, the tissue should contain 300 mg/kg in equilibrium. The measured tissue concentration for diazepam (figure 4) of 45 mg/kg certainly demonstrates that diazepam has

really concentrated in the tissue and is not simply water based equilibration with the dissolved solution. However, the accumulation in the fat of the whole cube of meat does not appear to be equilibrated. It may thus be concluded that the 24h loading time was insufficient to fully equilibrate the cube of meat, and that diazepam was most probably not homogeneously distributed in the tissue, which will impact the assessment of the SPME sampling in center of the tissue.

For amitriptyline, a 24h incubation of the tissue using 10 mg/L as loading concentration was chosen for all experiments. Figure 4A shows the resulting tissue concentrations for both diazepam and amitriptyline. Interestingly, tissue concentrations of amitriptyline (65 mg/kg) are higher than those for diazepam (45 mg/kg). Although amitriptyline is 99.6% cationic at pH 7.4 (pKa 9.76 *(ChemAxon, www.chemicalize.org)*), it absorbed high and fast despite the compound being predominantly in the charged form. Neutral amitriptyline has a $\log K_{ow}$ of 4.92, and the small 0.4% of neutral species may readily permeate through the cell membranes of the muscle tissue, speciating again inside the muscle cells. The cationic amitriptyline species have a measured phospholipid affinity ($\log K_{PLIPW,ion}$) on immobilized artificial membrane HPLC column (IAM-HPLC) of 3.34-3.9 L/kg (both determined at pH 5, first value from *(Vrakas et al. 2006)*, latter *(Droge, pers. comm.)*), and 3.4 L/kg in experiments with solid supported lipid membranes (TRANSIL-SSLM, *(Sovicell)*), which is higher than the liposomal affinity of diazepam. Amitriptyline was thus expected to have a relatively high partitioning compared to diazepam to the tissue, but amitriptyline also showed an ease of passive permeation into the tissue despite the large ionic fraction. As for diazepam, the measured tissue concentration for amitriptyline based after 24h loading appears to be below the equilibrium concentration, although there are other factors that might influence partitioning to tissue of cationic amitriptyline, such as binding to various structural tissue proteins and particularly to phospholipid cell membranes. Binding constants of amitriptyline to albumin (HSA) and α_1-acid glycoprotein (AGP) are 2.1 L/kg *(Peltenburg et al. 2015a)* and 4.3 L/kg *(Chrysanthakopoulos et al. 2014)*, respectively, although these proteins are plasma proteins and not the structural proteins present in muscle tissue. To aid in experimental work with passive sampling on tissue distribution, much more data is needed on fiber-tissue distribution coefficients for different tissue types and compounds. A possible solution was suggested by *(Zhang et al. 2010)*, to build a database that lists distribution coefficients for common fiber coatings, different tissue types, different species of animals and different

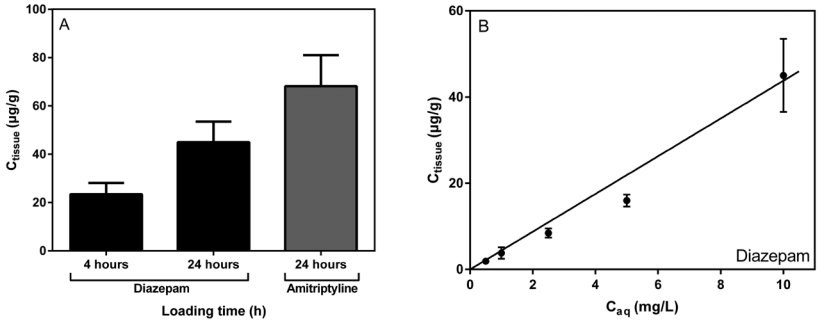

Figure 4. Tissue loading of diazepam and amitriptyline. Tissue was exposed to spiked PBS. A) Exposure concentration is 10 mg/L. Diazepam was loaded for 4 or 24h, amitriptyline only for 24h. B) Loading of diazepam at different concentrations. Loading interval is always 24h.

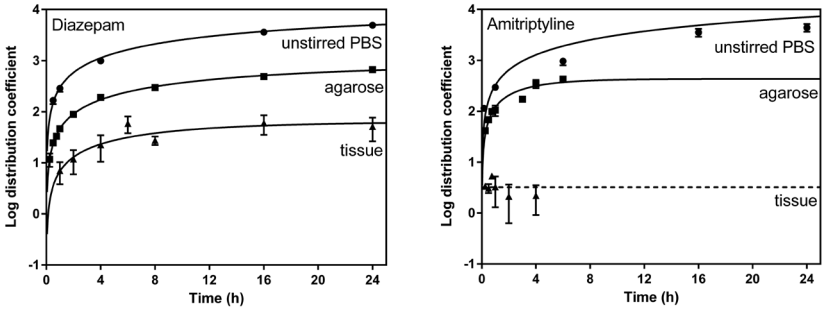

Figure 5. Uptake curves of diazepam and amitriptyline to the C18/SCX fiber from unstirred PBS, agarose gel and tissue. Exposure of the fibers is static, so no form of agitation was employed. PBS curves for diazepam and amitriptyline include data points up to 144 h and 336 h, respectively. Please note that for sampling from agarose gel and tissue, only data points are available with shorter sampling times than the calculated equilibration time. Uptake of amitriptyline from unstirred PBS is reprinted with publisher permission from the Supporting Information of *(Peltenburg et al. 2015b)*.

compounds. Future studies could determine in detail to which depth both compounds had permeated in the cube of tissue, and a more complete loading curve within a feasible period of time (while avoiding tissue decomposition) to assess whether the compound is fully equilibrated in the tissue. A thinner slice of tissue is likely to equilibrate faster, facilitating the conclusions from the SPME sampling.

Equilibration times in PBS, agarose gel and tissue

For both diazepam and amitriptyline, uptake curves were made for sorption of the compounds from loaded tissue into the SPME fiber (figure 5). For comparison, uptake curves in PBS and agarose gels were included. For both compounds, sorption to the C18/SCX coating from PBS is highest, followed by agarose gel and tissue. This is not unexpected, as sorption affinity is based on the difference in polarity or hydrophobicity between the sample matrix and the fiber coating. Sorption from PBS, a highly polar and hydrophilic environment, is highest as the phase difference between PBS and the coating is large. Agarose gel and tissue have more hydrophobic properties compared to PBS, and thus are able to trap or sorb a chemical more strongly.

For diazepam, equilibration time ($t_{95\%}$) from unstirred PBS is 89 hours, while this is 56 hours for agarose gel, both with static fiber exposure. Interestingly, uptake from tissue reaches equilibrium after 26 hours. Regarding the distribution coefficient and the time to equilibrate the SPME fiber, agarose gel does not appear to be a good replacement for actual tissue, but it has to be kept in mind that the tissue loading was probably not fully equilibrated. Furthermore, we use 1% agarose gel, where it was previously reported that rate constants for agarose gels and fish muscle tissue were most similar using 0.8% agarose gels *(Togunde et al. 2012a)*. For amitriptyline, $t_{95\%}$ from unstirred PBS is 326 hours, while this is 11 hours for agarose gel. For tissue, no equilibration time could be calculated as all exposure times used reach the same fiber-tissue distribution.

Uptake from a agarose gel phase and tissue into the fiber is a complex phenomenon. In an aqueous solution, uptake into the fiber is often determined by diffusion in an aqueous diffusion layer surrounding the fiber coating and the rate of uptake is then determined by a diffusion coefficient and thickness of the aqueous boundary layer. We don't have insight into a diffusion layer surrounding the fiber in the agarose gel or in the tissue. Uptake may occur via the aqueous phase in tissue or gel or it may be related to direct contact between

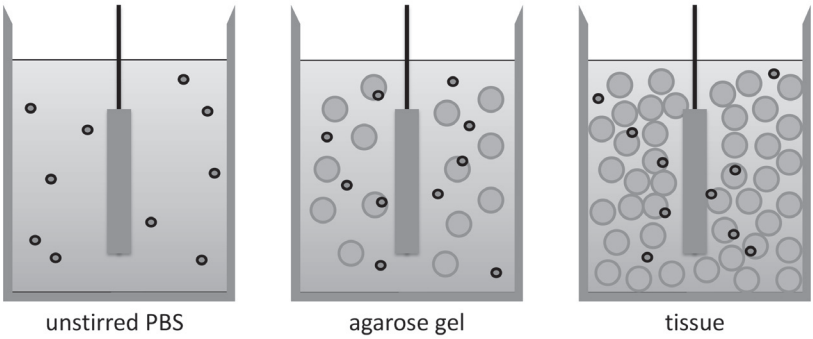

Figure 6. Graphical representation of mass transfer processes in unstirred PBS, agarose gel and tissue. In unstirred PBS, all molecules are free and diffusion is not limited. In agarose gel, there is some binding of the test compounds diazepam and amitriptyline, but depletion of free compound close to the fiber can be replenished by release of bound compound (facilitated transport). For tissue, this binding is even higher, which again can cause facilitated transport. However, the uptake may also occur due to direct contact between the fiber and the tissue.

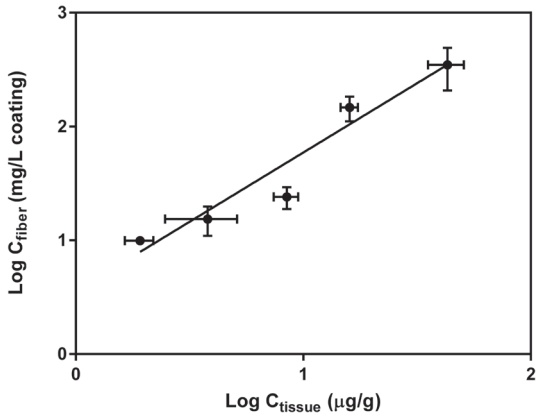

Figure 7. Concentration dependent sorption of diazepam from tissue. Loading time was always 24h, fibers were exposed for 2h. Tissue was loaded in triplicate for different PBS concentrations, hence the large standard deviation over the x-axis.

Loading concentration (mg/L)	Fiber concentration (mg/L coating)	Tissue concentration (µg/g)	Distribution coefficient (log D_{ft})
0.5	9.92 ± 0.85	1.91 ± 0.27	0.72 ± 0.03
1.0	15.4 ± 4.41	3.80 ± 1.33	0.61 ± 0.03
2.5	24.0 ± 5.23	8.46 ± 1.04	0.45 ± 0.05
5.0	147 ± 35.8	16.0 ± 1.40	0.96 ± 0.13
10.0	349 ± 141	43.1 ± 7.70	1.03 ± 0.21

Table 1. Sorption of diazepam from tissue at different loading concentrations.

Fiber concentrations, tissue concentrations and the corresponding distribution coefficient are given, including standard deviation of a triplicate measurement. Distribution coefficient of 2.5 mg/L loading concentration is significantly different than those of 0.5 mg/L and 1.0 mg/L.

tissue and fiber coating. If we assume that there is an aqueous diffusion layer surrounding the fiber, a process such as facilitated transport may explain the shorter equilibration time in agarose and tissue. Facilitated transport in fiber uptake has been observed in aqueous solutions that contain proteins where desorption of compound in the diffusion layer leads to an additional flux of the unbound molecule to the fiber coating. Agarose and tissue may have the same effect, as a compound will also be sorbed to these two phases. Because we do not have a clear view on the actual processes, these explanations for the differences in equilibration times remain speculative. Figure 6 is a graphical representation of these potential uptake processes.

SPME sampling in loaded tissue

To study concentration dependent sorption from tissue, tissue was loaded using different diazepam concentrations, leading to different (and probably inhomogeneously distributed, see above) tissue concentrations (figure 3B). Fibers were exposed to loaded tissue for 2 hours. Even though 2h is well below the equilibration of the SPME fiber with the concentration in the tissue, there is a quite good linear relationship between tissue and fiber concentrations. Figure 7 shows the sorption isotherm between tissue and fiber concentration that ultimately, under better defined tissue loading, may be used as an adequate calibration curve that directly relates SPME measurements with tissue concentrations.

Using this data, distribution coefficients for tissue (log D_{ft}) can be calculated for each loading concentration (table 1). This also shows deviations from a single distribution constant. However, log D_{ft} values are within 0.5 log units of each other at all concentrations. Only the log D_{ft} for the loading concentration 2.5 mg/L is significantly different from the other distribution coefficients. This can be attributed to a lower than expected fiber concentration at that loading concentration.

Clearly, sorption from agarose gel to the C18/SCX fiber and from tissue to the C18/SCX fiber is not equal (figure 5). Tissue contains various protein types and cell membranes and other organic structures, that can bind chemicals and which make tissues in general much more heterogeneous than agarose gels. Agarose gel could be modified to be more similar to tissue, for instance by using proteins such as BSA *(Jiang et al. 2015)* and liposomes. However, the development of artificial tissue is out of the scope of this paper, as we only used agarose as a model for hindered diffusion *(Muhr & Blanshard 1982)*.

CONCLUSION

This paper shows a detailed evaluation of the C18/SCX fiber as a direct sampling tool in semisolid tissue. Neutral diazepam and largely cationic amitriptyline were used as test compounds, to show that this fiber can efficiently extract both neutral and cationic compounds. Agarose gel was used as a tissue surrogate, to simulate changed rates of diffusion and tortuosity of the matrix. Linear sampling isotherms were observed for agarose gel. The results with tissue were more complex, as the cubes of muscle meat were difficult to equilibrate to a homogeneous loading concentration in our applied test systems. This influenced our sampling kinetics and extraction linearity with unknown uncertainty. Still, the C18/SCX fiber extracted both diazepam and amitriptyline from the muscle tissue, and at higher diazepam concentrations in the tissue also higher amounts were extracted with the sampler. The proposed SPME method seems feasible for tissue sampling, and seems already of good use for qualitative screening of all kinds of toxicants in tissue, as seen in the obtained sorption isotherm between tissue and fiber concentration. However, correct calibration of fiber extracts requires more effort in order to facilitate quantitative measurements. In future research, such quantitative measurements are required to be able to apply the current SPME methodology in forensic toxicology.

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7

GENERAL DISCUSSION

CONTEXT

The focus of this thesis is to develop a sampling method to study postmortem redistribution. Firstly, postmortem drug redistribution as a phenomenon is complex to study, as postmortem physiological changes lead to a difference in drug behavior compared to the antemortem situation. One essential postmortem change is a decrease in body pH, which results in a change in ionization state for ionizable chemicals. Especially cationic compounds seem to be prone to postmortem redistribution. As many pharmaceuticals and drugs are bases, these are the main analytes studied here.

Secondly, to be able to study postmortem redistribution in time, i.e. by incorporating repeated sampling, the method needs to be non-disturbing and based on an extraction of a chemical that does not affect the concentration. This resulted in choosing solid-phase microextraction (SPME) as the desired method, as a fast and simple sampling tool. Extractions using SPME can be performed in a negligible depletion mode. The SPME method employs a novel prototype coating based on C18 and strong cation exchange groups, which is specifically able to extract cationic compounds.

The discussion is presented in four parts. First, the sorption of cationic compounds to the C18/SCX fiber is discussed (based on Part I; Chapter 2-4). Secondly, using literature data on sorption constants for cationic compounds to different tissues, the *in vivo* distribution of these types of compounds can be modeled. The distribution of chemicals depends on various sorption processes (e.g. binding to protein, membrane and tissue). The third part of the discussion is an assessment of the use of SPME to study these sorption processes (based on Part II; Chapter 5 & 6). Finally, the potential of SPME to study postmortem redistribution will be discussed.

SORPTION OF CATIONIC COMPOUNDS TO C18/SCX SPME FIBERS

After the development of solid-phase microextraction (SPME) as sampling tool, it was initially used to sample volatile organic compounds from the headspace of the sample (i.e. above the liquid matrix) *(Arthur & Pawliszyn 1990)*. Volatility is dependent on vapor pressure and aqueous solubility (summarized in the Henry's law constant). To force compounds into the headspace, salts can be added to decrease the solubility of a compound and the pH can be adjusted so that compounds become neutral *(Buchholz & Pawliszyn 1994)*. The advantage of headspace sampling is that there is no contamination from non-volatile chemicals present in the aqueous phase. However, if a compound has a low Henry's law constant and thus low volatility, the effectiveness of headspace SPME sampling is low. In that case, the SPME fiber can be directly placed in the aqueous phase (direct immersion).

To sample ionized compounds without adjusting the pH, direct immersion of the SPME fiber is required as these compounds are not volatile. However, early SPME coatings were still only able to sorb the neutral fraction of ionizable compounds *(Risticevic et al. 2010)*. Again, the addition of salts and the adjustment of pH to obtain a less soluble, neutral compound increases extraction yield, for instance when sampling phenolic compounds using polyacrylate fibers *(Buchholz & Pawliszyn 1994)*. However, sample modifications might not always be desirable or possible in all sampling scenarios.

To still obtain high extraction yields for ionizable compounds, new SPME coatings were needed that are capable of sorbing the ionized form of these compounds. Increased sorption of cationic compounds was described using electrochemically enhanced SPME, where a negative potential is applied to the fiber to increase extraction efficiency *(Zeng et al. 2011)*. Other sampler coatings were shown to contain negatively charged groups, thereby effectively sorbing cationic compounds. Polyacrylate (PA) fibers containing carboxylate groups were capable of sorbing cationic surfactants up to a total cation exchange capacity of 30 mmol/L coating *(Chen et al. 2012)*. Ion-exchange membranes containing sulfonic acid groups showed high sorption of three structurally diverse cations (difenzoquat, nicotine and verapamil), up to a cation exchange capacity of 1200 mmol/kg *(Oemisch et al. 2014)*. Another potentially promising SPME coating is extensively described in this thesis: the C18/SCX fiber.

Comparison of the C18/SCX fiber with other SPME fibers

The C18/SCX fiber consists of hydrophobic octadecyl chains (C18) and propylsulfonic acid acting as strong cation exchange groups (SCX). These fibers show increased affinity for amphetamine compared to polyacrylate (PA) fibers (Chapter 2). PA fibers mainly sorb the neutral fraction of ionizable compounds but also contain negatively charged carboxylate groups at pH>3 (pKa 4.7 *(ChemAxon, www.chemicalize.org)*), resulting in a pH dependent sorption affinity of cationic compounds. Amphetamine shows increased affinity for the C18/SCX fiber over PA at any pH (table 1 & Chapter 2). For both coatings, sorption affinity of the neutral species of amphetamine (pH 11.4) is higher than that of the cationic species (pH 7.4). However, for the PA fiber, there is a clear difference in sorption between pH 3.1 and pH 7.4. As amphetamine is >99% cationic in this pH range (pKa 9.9, *(ChemAxon, www.chemicalize.org)*), the lower sorption affinity at pH 3.1 can be attributed to the protonation of the carboxylic groups in the polyacrylate. The C18/SCX fiber contains sulfonic acid groups with a reported pKa of <1, making these groups negatively charged over the entire pH range. Despite this, there is still a small increase in sorption affinity between pH 3 and pH 7.4. This can either be attributed to competition of H^+ for the strong exchange groups (especially at low pH), or sorption to dissociated silanol groups in the coating (silanol pKa 6.8 *(Escher et al. 2000)*).

The C18/SCX fiber also showed increased affinity for amphetamine compared to analogous C18 fibers (table 1). Interestingly, the C18 fiber also shows high affinity for cationic amphetamine, although it contains no strong cation exchange groups. Again, dissociated silanol groups in the coating can contribute to the sorption of cationic compounds to the C18 fiber. To study the influence of these negatively charged groups, sorption of anionic diclofenac (pKa 4.0 *(ChemAxon, www.chemicalize.org)*) to the C18 fiber and the C18/SCX fiber was compared. Diclofenac sorbed equally as strong to the C18 and the C18/SCX fiber at pH 7.4, indicating that there is no repulsion of the anion by the SCX groups in the C18/SCX fiber (Chapter 4). However, sorption of diclofenac to the C18/SCX fiber was lower than predicted based on log D_{fw}-log K_{ow} relationship established using cationic compounds (Chapter 4). This could indicate that both the C18 and the C18/SCX fiber contain unfavorable sorption moieties for anionic compounds, which might be attributed to dissociated silanol groups. Likewise, the endcapping of silanol groups in C18-based SPME fibers can also contribute to sorption. For instance, reversed-phase alkylsilica HPLC columns carry a positive charge at low pH, related to the endcapping

	log D_{fw}		
SPME coating	**pH 3.1**	**pH 7.4**	**pH 11.4**
PA (7 µm)	-0.7	0.9	1.7
C18	1.9	2.3	2.4
C18/SCX	2.1	2.5	2.5

Table 1. Sorption affinity (log D_{fw}) of amphetamine for polyacrylate, C18 or C18/SCX fibers at different pH. Data from Chapter 2.

of silanol groups with positively charged moieties *(Loeser 2008, Marchand & Snyder 2008)*. Although only one anionic compound was tested, C18 and C18/SCX SPME fibers appear to be capable of sorbing anions, thereby increasing their chemical applicability domain.

The comparison between the C18 and C18/SCX fiber learns that the latter shows somewhat higher sorption affinities for cationic compounds. This can be ascribed to the strong cation exchange sites present in the C18/SCX fiber. At pH 7.4, differences in sorption affinity between C18 and C18/SCX fibers were 0.2 log units for amphetamine, 0.5 log units for amitriptyline and 0.7 log units for trimethoprim (Chapter 3). This shows the benefit of the strong cation exchange sites in the C18/SCX fiber. However, sorption affinity of the neutral fraction of these compounds to the C18/SCX fiber is still higher than for the cationic species (Chapter 3). For sorbents that only possess cation exchange sites (such as soil organic matter *(Droge & Goss 2012)*, humic acids *(Karthikeyan & Chorover 2002, Sibley & Pedersen 2008)*, and liposomes *(Hunziker et al. 2001)*), sorption affinity is higher for cationic species compared to neutral species. As the C18/SCX fiber shows higher sorption affinity for neutral species compared to cationic species, this shows the benefit of the C18 moiety in this fiber.

Effects of the presence of other cations on sorption

As can be expected for the sorption of charged compounds to charged coatings, competition effects can occur in the presence of other (in)organic cations. The effects of Na^+, K^+ and Ca^{2+} on sorption of amphetamine to the C18/SCX fiber were tested (Chapter 2). This showed clear competitive effects of these inorganic cations, as was previously also shown for other sorbents based on cation exchange *(Chen et al. 2012, Droge & Goss 2012, Karthikeyan & Chorover 2002, Sibley & Pedersen 2008)*. Interestingly, bivalent ions showed similar competitive effects to monovalent ions at equal ionic strength, which contradicts with other findings where Ca^{2+} reduced sorption affinity with 1

log unit more than Na^+ at equal ionic strength *(Chen et al. 2012, Droge & Goss 2012)*. However, the effect of competing cations is expected to be larger for compounds with a low sorption affinity (such as amphetamine). The influence of realistic changes in ionic strength and composition, as they might occur postmortem, was studied using amphetamine and amitriptyline (Chapter 3). This showed only marginal competitive effects in the postmortem condition for both compounds, indicating that sorption mainly occurs via adsorption to the C18 surface. However, the salinity effects observed most likely result from salt-induced changes in the existing electrical double layer generated by the SCX groups. These changes, although marginal, should be considered when sampling postmortem.

Competition effects do not only occur with inorganic cations, but also for larger organic cations. Binary mixtures of amphetamine and amitriptyline showed a decrease in sorption affinity of amphetamine when high concentrations of amitriptyline were added (Chapter 3). As amitriptyline has a much higher sorption affinity for the C18/SCX fiber than amphetamine, only the sorption affinity of amphetamine is decreased. Competition effects only occurred when the total fiber loading exceeds 10 mmol/L. The data in this thesis has consistently shown that sorption of any compound is linear up to at least 1 mmol/L, after which leveling off to a maximum (Chapter 4). This maximum can be termed the cation exchange capacity of the fiber, although adsorption to the C18 surface is also a competitive process with a finite number of sorption sites. This cation exchange capacity (CEC) or maximum fiber concentration was first reported as 47 mmol/L based on amphetamine data (Chapter 2), later as at least 120 mmol/L based on amitriptyline data (Chapter 3), and finally as 400 mmol/L based on chlorpromazine data (Chapter 4). Discrepancies in these numbers are caused by the fitting of experimental data using the Langmuir sorption isotherm (equation 8, Chapter 1). In comparison, ion exchange membranes used to extract cationic compounds have a CEC of 1200 mmol/kg, although this material is not capable to sorb large compounds (>300 Da) *(Oemisch et al. 2014)*.

Modelling of sorption

Sorption isotherms are linear up to 1 mmol/L, and then level off before reaching a maximum. Attempts to fit sorption data with the Langmuir equation (equation 8, Chapter 1) showed that sorption started to become nonlinear well below 10% of the CEC (Chapter 2) when sampling from PBS. The Langmuir equation did fit well for sorption of amphetamine from pure

water, indicating that the addition of inorganic salts changes the electrical double layer established by the SCX sites in the fiber. This is also supported by good Langmuir fits for the neutral benzodiazepines diazepam, temazepam and oxazepam, as these are not influenced by ionic strength (Chapter 4). In this thesis, sorption isotherms are often fitted using the Freundlich equation (equation 7, Chapter 1) and sorption affinity is calculated at fiber concentrations of 1 mmol/L, as this is in the linear range for all tested compounds.

Using the calculated sorption affinities of ten C_xH_yN cations, a clear relationship was found between sorption affinity and log K_{ow} for compounds without H-bonding capacity (Chapter 4). Interestingly, polar compounds that possess the ability to form H-bonds show a higher sorption affinity than predicted based on this log K_{ow} relationship. This is surprising as the C18 moiety in the C18/SCX fiber does not sorb polar compounds well, unlike octanol which is capable of forming H-bonds with polar compounds. The influence of H-bond donor or acceptor moieties should be investigated further to gain a detailed insight into their contribution to sorption to the C18/SCX fiber.

In summary

The data collected in Chapter 4 will contribute to the prediction of sorption affinities of cationic compounds to the C18/SCX fiber. Overall, the C18/SCX fiber appears to have a large chemical applicability domain, that includes ionizable compounds (both cationic and anionic) and polar organic chemicals.

IN VIVO DISTRIBUTION OF CATIONIC COMPOUNDS

Studying sorption or binding of compounds in the body is important, as binding to blood components and tissue determines the free concentration of a compound. Only the free drug concentration *in vivo* interacts with the target site and exerts an effect. This free concentration is therefore an important measurement in pharmacology and toxicology. The interactions of the free drug concentration with its environment are presented in two parts *(Smith et al. 2010)*. The first part states that the free concentration at the site of action is the species that exerts pharmacological activity. For most compounds, this is well established and proven, for instance in the relationship between *in vivo* free concentrations and *in vitro* potency measured as IC50 values *(Smith et al. 2010)*. The second part of the free drug hypothesis states that, at steady state, equal free

drug concentrations exist on both sides of any biological membrane. This process is driven by passive diffusion, and is thus in part dependent on the existing gradient across the membrane *(Smith et al. 2010)*.

Current knowledge on body distribution of cationic compounds

While protein binding has been described as early as the 1930s, tissue binding has long been overlooked as a major contributor to the binding of drugs until the 1970s *(Fichtl et al. 1991)*. Several general principles have been drawn up for binding of drugs to proteins and tissue, but these principles are generally driven by physical-chemical properties that are well defined for neutral compounds, such as log K_{ow}. The behavior of neutral organic chemicals can be predicted accurately, for instance their ability to accumulate in membrane lipids *(Endo et al. 2011)* or their behavior in *in vitro* systems *(Armitage et al. 2014, Groothuis et al. 2015)*. However, it is still relatively unclear how ionized compounds interact with plasma proteins or cell membranes, and, more importantly, how this interaction can be predicted or modeled.

Binding of cationic compounds to cell membranes has been described in several publications *(Austin et al. 2005, Howell & Chauhan 2009, Vrakas et al. 2006)*. In an octanol-water system, ionizable compounds clearly show a favorable distribution to the octanol phase in their neutral form. However, in a membrane-water system, partitioning of the ionized fraction is clearly enhanced compared to the octanol-water system *(Avdeef et al. 1998)*. Ionic compounds show higher sorption to membranes, as they have a favorable binding in both their neutral and their charged form *(Avdeef et al. 1998, Escher & Schwarzenbach 1996)*. *(Avdeef et al. 1998)* proposed how ionizable compounds behave inside a membrane. In neutral form, an ionizable compound will position itself inside the membrane with its hydrophobic part alongside the acyl chains inside the bilayer and its hydrophilic part near the surface of the membrane. In charged form, the compound will reposition towards the exterior of the membrane. Then it first encounters negatively charged phosphate groups, where cationic compounds will remain due to electrostatic interactions. Anionic compounds will move even further to the surface of the membrane, until they reach the positively charged trimethylammonium groups. As a compound moves more towards the surface of the membrane, membrane affinity will decrease which is why cations usually have higher membrane affinity than anions.

Binding of drug molecules influences both pharmacodynamics as well as pharmacokinetics. Binding to receptors is necessary for an effect, whereas binding to carrier proteins and enzymes determines the free fraction of the drug that is available for binding to these receptors. For some compounds, large amounts can be bound to plasma proteins and tissue constituents without pharmacological effect, thereby determining the drug distribution throughout the body and with that a drug's concentration-time curve *(Fichtl et al. 1991)*. In drug discovery and drug development, special focus lies in determining the plasma protein binding of a compound, and structural drug modifications can be made to optimize the free fraction based on this outcome. However, little attention is paid to membrane or tissue binding, while this may be a significant factor influencing free concentrations.

Plasma protein binding occurs mainly through two different proteins. Human serum albumin (HSA) is the most abundant protein in blood. Two dominant binding sites are responsible for most of all drug binding, although hydrophobic and electrostatic interactions to other sites are also possible *(Ghuman et al. 2005)*. Another important plasma protein is α_1-acid glycoprotein (AGP). AGP is known for binding basic compounds, although not all basic compounds show high protein binding and also neutral and acidic compounds can show binding to AGP *(Israili & Dayton 2001)*. Antemortem, protein binding can vary interindividually, as protein concentrations can vary with age and with various diseases *(Viani et al. 1992)*.

It is clear that there are several binding phases *in vivo* that influence the freely dissolved concentration of a chemical. However, the body distribution of a chemical is highly specific, especially for cationic compounds. This section focusses on affinities of (mainly cationic) compounds to different binding phases. These affinities are compared directly and translated to bound fractions in the human body. This basic model should then be able to answer the question on whether membrane binding should always be included for cationic compounds, or whether the contribution of membrane binding is limited in comparison to protein binding.

Compound	pKa	Log K_{ow}	K_{HSA} (L/kg)	K_{AGP} (L/kg)	$K_{membrane}$ (L/kg)
Acebutolol	9.57	1.95	1.18	n.s.	1.90
Alprenolol	9.67	2.88	1.09	3.03	2.79
Amitriptyline	9.76	4.92	2.18	4.35	3.84
Amlodipine	9.45	4.16	3.74	3.89	3.84
Atenolol	9.67	0.10	0.35	n.s.	1.09
Bupivacaine	8.00[a]	3.64	2.06	4.12	2.45
Chlorpheniramine	9.47	3.39	1.69	3.02	2.59
Chlorpromazine	9.20	5.20	2.76	4.32	3.94
Clomipramine	9.20	5.39	2.64	4.28	3.50
Desipramine	10.02	4.13	2.65	3.71	3.48
Diazepam	2.92[b]	2.91	3.26	3.10	2.76
Diltiazem	8.18[a]	3.63	1.63	3.41	3.04
Diphenhydramine	8.87	3.66	1.50	3.73	2.90
Haloperidol	8.05[a]	3.01	2.26	3.17	3.43
Imipramine	9.20	4.80	2.19	3.80	3.35
Labetalol	9.80	2.31	1.11	2.73	1.84
Lidocaine	7.75[a]	3.63	1.71	3.20	1.45
Metoprolol	9.67	1.79	1.84	n.s.	1.66
Nicardipine	8.18[a]	5.13	2.33	4.09	3.89
Nortriptyline	10.47	5.65	1.99	3.94	3.67
Oxprenolol	9.67	2.29	1.03	3.56	2.15
Pindolol	9.67	1.97	0.92	2.92	2.10
Promazine	9.20	4.63	2.81	3.82	3.84
Promethazine	9.05	4.78	2.56	4.35	3.33
Propranolol	9.67	3.10	1.75	3.57	2.87
Quinidine	9.05	3.44	1.68	3.48	2.30
Thioridazine	8.93	6.13	3.14	5.13	3.81
Trifluprom azine	9.20	5.70	2.74	4.79	4.58
Verapamil	9.68	3.90	1.64	3.52	3.47
Warfarin	5.56[c]	3.42	2.83	3.57	1.40

Table 2. Overview of binding affinities for all study compounds.
pKa values are from *(ChemAxon, www.chemicalize.org)*, log K_{ow} are from ACD labs *(ChemSpider, www.chemspider.com)*. Binding affinities are averaged when multiple values were available. n.s. indicates values where protein binding was measured but found to be not significant (approximating zero).

[a] All compounds are >97% cationic at pH 7.4, with exception of bupivacaine (80% charged), diltiazem (86%), haloperidol (82%), lidocaine (69%) and nicardipine (86%).

[b] Diazepam has an ionizable group, but is 100% neutral at physiological pH.

[c] Warfarin is >98% anionic at pH 7.4.

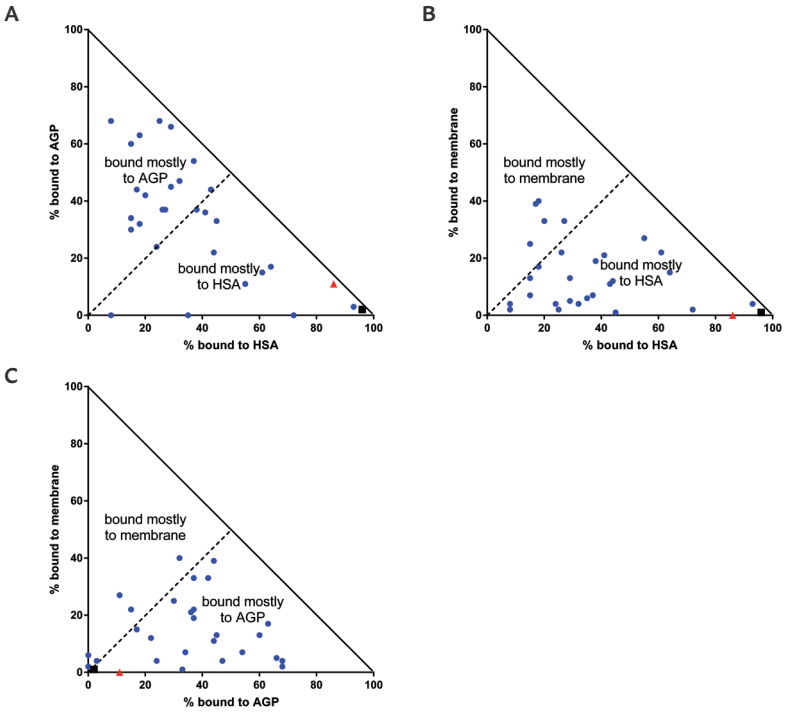

Figure 1. Percentage of the test compounds bound to the different binding phases. Total of the different percentages can never exceed 100%, as indicated by the solid lines. Dotted lines indicate equal bound fractions to both binding phases. Diazepam is indicated as a black square, warfarin as a red triangle. A) % bound to HSA vs % bound to AGP, B) % bound to HSA vs % bound to membrane, C) % bound to AGP vs % bound to membrane.

Binding affinities of cationic compounds to proteins and membrane

To compare binding affinities of different compounds to HSA, AGP and membranes, we collected and summarized data for 28 cationic compounds, one neutral compound (diazepam) and one anionic compound (warfarin) (table 2).

A simple comparison of the literature data in table 2 shows that for most cationic compounds, binding affinities are in the following order: log K_{AGP}>log K_{mem}>log K_{HSA}. For neutral diazepam, the order is log K_{HSA}>log K_{AGP}>log K_{mem}, while for anionic warfarin this is log K_{AGP}>log K_{HSA}>log K_{mem}. Obviously, it appears that membrane binding has a larger contribution to the bound fraction of cationic compounds than to the bound fraction of neutral or anionic compounds.

Though binding affinities give a good idea of the relative degree of binding of a compound, they do not incorporate the absolute concentration of a binding phase in the body. Using binding affinities and the concentration or mass of the different binding phases present in the body, free and bound fractions of all compounds to the different binding phases in human blood can be determined. To calculate this, we assumed a compartment of 1 L, with 1 mg of compound added. The amount of the different binding phases in 1 L of blood was calculated. For HSA, this is 40 grams assuming the most commonly used concentration in literature (40 g/L or 600 µM). For AGP, we used a concentration of 0.9 g/L (23 µM) for the calculations. For membrane, we used the number of red blood cells in 1 L ($5{\cdot}10^{12}$ cells/L, individual weight 27 pg *(Phillips et al. 2012)*) and assumed a phospholipid content of 1% based on *(Schmitt 2008)*. This results in an amount of 1.35 grams of membrane per liter of blood. We excluded leukocytes and platelets as contributors to total membrane volume, as these blood cells are greatly outnumbered by the amount of red blood cells.

Figure 1 shows the percentage bound of the test compounds to HSA, AGP and membrane. When comparing AGP and HSA (figure 1A), it is clear that most cationic compounds show higher protein binding to AGP than to HSA. As the concentration of HSA is 40 times higher than that of AGP, binding affinity of AGP needs to be 1.6 log units higher than that of HSA for AGP to become the dominant binding phase for any compound. A comparison between binding to HSA and membrane (figure 1B), shows that bound fraction to membrane

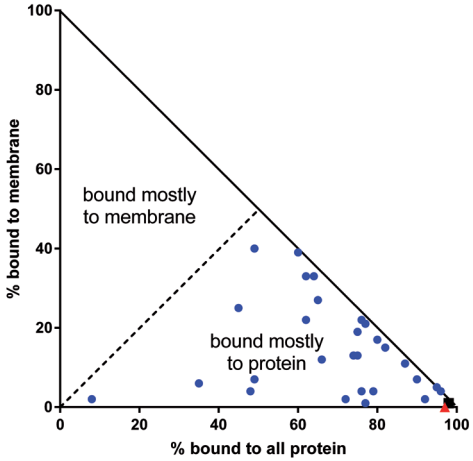

Figure 2. Percentage of the test compounds bound to total protein (HSA and AGP) versus bound to membrane. Total of the different percentages can never exceed 100%, as indicated by the solid line. The dotted line indicates equal binding to both binding phases. Diazepam is indicated as a black square, warfarin as a red triangle (both in the lower right hand corner).

are higher for 5 out of 28 cationic compounds (alprenolol, nicardipine, nortriptyline, triflupromazine and verapamil). Again, the concentration of HSA is around 30 times higher than that of membranes in blood, so membrane only becomes the dominant binding phase when its binding affinity is 1.5 log units higher than the binding affinity for HSA. AGP and membrane are present in similar concentrations. Bound fractions therefore follow the distribution of binding affinities, and in general binding to AGP is higher than to membrane (figure 1C). Only a few compounds bind stronger to membrane than to AGP (amlodipine, haloperidol, promazine and verapamil).

To truly show the contribution of membranes in the total bound fraction of cationic compounds, bound fractions to proteins were put together and plotted against membrane binding (figure 2).

When comparing total protein binding to membrane binding (figure 2), it is clear that membrane binding only has a limited contribution to total binding of a cationic compound in blood. It does appear that the contribution

of membrane binding is somewhat higher for cationic compounds than for neutral or anionic compounds. Diazepam and warfarin plot in the lower right hand corner (i.e. very little membrane binding but high protein binding), whereas the cationic compounds in general show a higher contribution of membrane binding to the total bound fraction. However, more data on neutral and anionic compounds should be included to further study this observation. The proposed model to study distribution should also be explored further, as it currently only represents binding in human blood without incorporation of tissue binding. Some considerations on tissue binding will be discussed in the following paragraph.

Other factors contributing to tissue distribution

The proposed distribution of cationic compounds is only relevant in human blood. To study whole body distribution of cationic compounds (or any compound), distribution into tissue needs to be incorporated. There are several factors that need to be considered to determine the contribution of tissue binding to whole body distribution.

First of all, there are some exceptions to the hypothesis that the free drug concentration is the most relevant entity in efficacy or toxicity, as described by *(Smith et al. 2010)*. This is not only limited to effect (dynamic phase), but also involves distribution and the kinetic phase. The effect of a drug can be unrelated to free concentration when a drug binds irreversibly to its target, thereby making the target unavailable for further binding or activation. Secondly, there are some exceptions to the establishment of an equilibrium between free concentration on either side of a membrane. Passive diffusion is hindered when a drug has a low passive permeability or when the tissue has limited blood flow, resulting in a very slow onset of steady state or the absence of steady state. On the other hand, several membrane transporters exist that can enhance either the influx or the efflux of a compound into or from the tissue, thereby overruling passive diffusion. These processes can have a profound influence on the distribution of a compound.

Distribution phenomena can be different for ionized compounds compared to neutral compounds. The main factor influencing the distribution of ionized compounds is pH. As mentioned before, the permeability of ionic species in for instance the intestine is much lower than for neutral species *(Avdeef 2012)*. For example, the transport of neutral alfentanil (base, pKa 7.5 *(ChemAxon, www.chemicalize.org)*) was 150-fold faster over a Caco2-cell monolayer than

for its cationic form *(Palm et al. 1999)*. However, the cationic form is still able to diffuse over the monolayer, and its contribution is even significant when the neutral fraction is <1% *(Palm et al. 1999)*.

Another factor contributing to the distribution of ionizable compounds is differences in intracellular pH. Cell pH can range between pH 4.5 in lysosomes and pH 8.0 in mitochondria *(Asokan & Cho 2002)*. For basic compounds, this can lead to a phenomenon called ion trapping (figure 3). Here, a neutral base can become ionized when reaching the lysosomes, where it is trapped as it becomes ionized due to the low pH inside the lysosome *(Kornhuber et al. 2010)*.

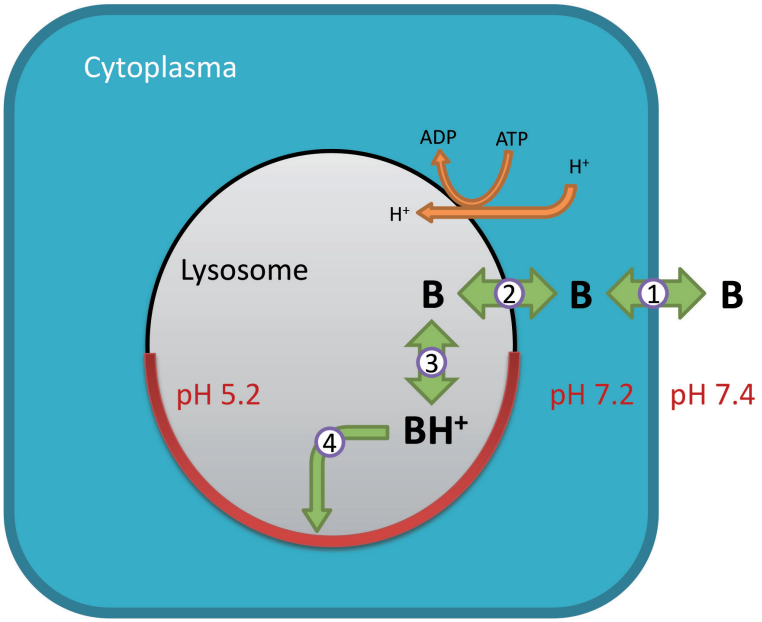

Figure 3. Ion trapping of a cationic compound. 1) The neutral fraction of a basic compound (B) partitions into the cell. 2) Further permeation of B into the lysosome. 3) The lower pH inside the lysosome leads to protonation of the base to its cationic form (BH^+). 4) The cation still has lipophilic properties, and accumulates in the lysosome membrane, thereby changing membrane permeability and leading to toxic effects. Adapted from *(Kornhuber et al. 2010)*.

The high accumulation of basic compounds can even lead to toxicokinetic effects. This occurs for instance in green algae, which show a high baseline toxicity to compounds with an aliphatic amine moiety *(Neuwoehner & Escher 2011)*.

Tissue binding can be driven by different biological binding phases, including proteins, and membrane and storage lipids. Biological membranes contain a variety of charged or ionizable lipids, including phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and phosphatidic acid *(Escher & Sigg 2004)*. Most of these membrane lipids are anionic at relevant physiological pH, while some are zwitterionic. Membrane affinities of ionized compounds can therefore change with membrane composition and with pH due to protonation of phospholipids *(Escher & Sigg 2004)*.

Whereas phospholipids are the main constituent of membranes, storage lipids are mostly made up of triacylglycerides *(Schmitt 2008)*. Differentiating between binding to membrane lipid or storage lipid can distinguish whether a compound is more likely to accumulate in membrane-rich tissue, such as the liver or kidney, or in fat tissue. For neutral compounds, binding affinities to membrane lipids can be up to two log units higher than for storage lipids, especially for compounds acting as H-bond donors as found by *(Endo et al. 2011)*. Although the authors stress that their work does not include ionized compounds, differences in binding affinities for membrane or storage lipids can be expected for these type of compounds as well.

In tissue binding, the protein fraction is usually viewed as a negligible phase compared to the total sorption capacity of the tissue *(DeBruyn & Gobas 2007)*. However, this contribution is in general between 1 and 10% that of lipid sorption for neutral compounds, with even higher contributions in lean tissues with less than 5% fat *(DeBruyn & Gobas 2007)*. Tissue proteins such as muscle protein and collagen are different proteins than the plasma proteins HSA and AGP. Binding affinities to muscle protein are structurally lower than binding affinities to HSA for neutral compounds *(Endo et al. 2012)*. Binding affinities of H-bond donor compounds to muscle protein can be similar to binding affinities to storage lipid *(Endo et al. 2012)*, showing that proteins should not be neglected when studying the total sorptive capacity of tissue.

In summary

Most studies on tissue binding have been performed on neutral compounds. This leaves the *in vivo* distribution of cationic compounds as still relatively little studied topic. Improved knowledge on this distribution will aid in the assessment of the bioaccumulation potential of cationic compounds, which is important for industry and governmental regulators.

APPLICATION OF C18/SCX SPME TO STUDY SORPTION PROCESSES

As already discussed in the previous section of this chapter, the free drug concentration at the site of action is the species that exerts pharmacological activity. By measuring free drug concentrations in a system, e.g. *in vivo* or *in vitro*, one could obtain useful information on the effective concentration in the system but also on the distribution of the compound and the effect of different binding or sorption processes.

Methods to measure free concentrations

Several methods exist to measure freely dissolved concentrations, usually with the intention to calculate binding affinities. The most commonly used methods include equilibrium dialysis, ultrafiltration and chromatographic methods. Downsides of these methods include binding to the apparatus and osmotic volume shift for equilibrium dialysis and ultrafiltration, as well as non-specific ligand binding, especially for ionized compounds, when using chromatographic methods *(Buscher et al. 2014)*. Another approach to measure freely dissolved concentrations is by using SPME. For instance, PDMS-coated SPME fibers were used to determine free concentrations of lidocaine in human plasma *(Koster et al. 2000)*. Here, the authors increased the extraction yield of basic lidocaine (pKa 7.8 *(ChemAxon, www.chemicalize.org)*) by increasing plasma pH to 9.5 to obtain a large neutral fraction of the drug *(Koster et al. 2000)*. Obviously, determining binding affinities at high pH lacks physiological relevance as binding affinities can differ at various pH, especially for ionizable compounds *(Musteata et al. 2006)*. Furthermore, by aiming for high extraction yields, a significant depletion of the free concentration occurs. This depletion can have a large effect on the existing equilibrium between bound and free and thereby on estimation of freely dissolved concentrations, which will be discussed later in this section.

SPME has often been applied *in vitro* or *in vivo* to determine freely dissolved concentrations and to study sorption processes. Many of these applications include environmental processes such as compound sorption to organic matter *(Droge & Goss 2013)*, organic carbon *(Haftka et al. 2013)*, sediment *(Rico-Rico et al. 2010)* and humic acid *(Holten Lützhøft et al. 2000)*. There are also several publications on pharmacological or toxicological applications of SPME, which study compound sorption to proteins such as albumin *(Broeders et al. 2011)* and chyme *(Oomen et al. 2000)*, but also to liposomes *(Escher et al. 2002)* and membranes *(Vaes et al. 1997)*.

Determining plasma protein binding using SPME

The number of bioanalytical applications of SPME is rapidly increasing. The main concerns when analyzing biological samples using SPME are a decrease in sensitivity due to competition by endogenous compounds, a decrease in free concentration available for uptake to the fiber, and high viscosity of the sample *(Musteata 2012)*. However, numerous papers have shown the great potential of SPME in bioanalytical applications. One of these applications is the determination of plasma protein binding of compounds. The premise of this application is simple, and described often (e.g. *(Musteata et al. 2006)*). An SPME fiber is exposed to a sample containing binding matrix, thereby sampling the free concentration. One should take potential depletion of the compound into account (see next section). To correlate the amount extracted by the fiber to the actual free concentration, an SPME calibration in PBS or any solution without binding matrix is needed. With spiked samples, the total concentration is simply the amount of drug added before exposure of the SPME fiber (or the remaining concentration in the sample after exposure of the fiber when significant depletion has occurred). For unknown samples, the total concentration can be determined using an SPME calibration in a matrix-matched solution (e.g. containing binding protein). When using both SPME and spectroscopic techniques in plasma protein binding studies, one could complement the quantitative results obtained with SPME with structural information on complex formation, using techniques such as protein fluorescence quenching and proton nuclear magnetic resonance imaging *(Bojko et al. 2012)*.

In this thesis, we studied freely dissolved concentrations of three cationic compounds (amitriptyline, amphetamine and tramadol) and one neutral compound (diazepam) using C18/SCX SPME fibers (Chapter 5). Using these freely dissolved concentrations, binding affinities of these compounds for

bovine and human serum albumin (BSA & HSA) could be calculated. We used physiological concentrations of the proteins, and added the compounds in concentrations ranging from (sub)therapeutic to toxic or lethal. Even for highly-protein bound cationic compounds at low (free) concentrations, the used SPME method showed good sensitivity (Chapter 5). For a correct calculation of the binding affinity, it is important to limit the analyte to protein ratio to a maximum of 0.1. This eliminates potential saturation of the binding sites present on the protein, and allows use of the total protein concentration when calculating binding affinity instead of the free protein concentration (Chapter 5). Using this approach, experimentally determined binding affinities were found to be very similar to literature values.

C18/SCX fibers were also directly employed in human plasma and human whole blood to study free concentrations (Chapter 5). We found clear differences between free concentrations in human plasma compared to HSA dissolved in PBS. Free concentrations for amphetamine and tramadol were higher in human plasma compared to HSA alone, while this was reversed for amitriptyline (Chapter 5). It is suspected that the presence of endogenous compounds such as fatty acids, hormones and bile acids, limits binding of low-affinity compounds such as amphetamine and tramadol in human plasma, but not in the HSA solution *(Peters Jr. 1995)*. For amitriptyline, binding to AGP is up to 400 times stronger than binding to HSA *(Hervé et al. 1998)*, explaining the decrease in free concentration in human plasma compared to HSA alone. For all cationic compounds, free concentrations were lower in human whole blood compared to human plasma (Chapter 5). This clearly shows binding to membrane or red blood cells *(Hinderling 1997)*, as also discussed extensively in the previous section. Especially for cationic compounds, binding affinities to membranes can be high, making membranes a dominant binding phase. For these compounds, determining free concentrations in whole blood might have more predictive value than in plasma.

Effect of depletion on calculated binding affinities

Important in studying free concentrations in any system, is that the free concentration is not depleted, thereby not disturbing the existing equilibrium between bound and free concentration. To ensure this, negligible depletion SPME (nd-SPME) was introduced *(Vaes et al. 1996)*. In nd-SPME, a negligible portion of the free fraction in the aqueous phase is extracted by the fiber. The maximum allowed depletion is usually set at 5% *(Heringa & Hermens 2003, Vaes et al. 1996)*. Besides negligible depletion, other prerequisites of nd-

SPME are an existing equilibrium between bound and free fraction and no effect of the binding matrix on the extraction. The latter includes both fouling and facilitated transport, both of which will be discussed in more detail later in this section. Another application of SPME to study freely dissolved concentrations is matrix SPME, which allows for a temporary depletion of the aqueous phase, as long as desorption from the matrix (i.e. bound drug becoming free) is instantaneous *(Mayer et al. 2000)*. Matrix SPME is usually employed at equilibrium, although sampling in the kinetic uptake phase is possible under certain conditions (Chapter 5).

Using diazepam, we studied the influence of depletion on calculated binding affinities (Chapter 5). Diazepam and protein concentrations were kept equal in each vial, but the sample volume was varied to allow for different degrees of depletion. After equilibrium exposure, we found identical binding affinities at all sample volumes, while depletion ranged between 4 and 47%. This clearly shows the principle behind matrix SPME: the depleted free concentration is replenished by release of bound drug. Interestingly, when depletion is significant, the same sample can be used to perform multiple extractions *(Musteata & Pawliszyn 2005)*. Here, the free (and thus total) concentration is depleted after a first extraction, leading to a new equilibrium between bound and free concentration. Multiple successive extractions can then be used to obtain multiple data points along the binding curve.

To further study the effects of depletion on calculated binding affinities, the experiment using different sample volumes was performed again using a pre-equilibrium exposure (i.e. in the kinetic uptake phase) (Chapter 5). Again, we found similar binding affinities at all sample volumes. This shows that the uptake kinetics to the fiber are not affected by the presence of the binding matrix, as this is one of the conditions that allows sampling in the kinetic uptake phase using matrix SPME. When the presence of binding matrix influences sampling, this is known as a matrix effect. Matrix effects will be discussed in detail later in this section.

Tissue sampling using SPME

The use of SPME as a direct sampling tool in tissue has gained special interest in recent years *(Ouyang et al. 2011)*. Most *in vivo* experimental work has been performed on the sampling of environmental contaminants, including pharmaceuticals, in fish *(Wang et al. 2011, Zhang et al. 2010, Zhang et al. 2012, Zhou et al. 2008)*. To overcome difficulties in sampling in tissue due to a

difference in density and porosity compared to aqueous matrices, agarose gel is often used as a tissue surrogate *(Togunde et al. 2012)*.

We studied differences in uptake kinetics for diazepam and amitriptyline to C18/SCX fibers in PBS, agarose gel and pork muscle tissue (Chapter 6). Uptake from agarose was fast for both compounds, indicating that diffusion in this system might not be as hindered as hypothesized. However, the agarose gel contains binding sites for the chemicals used here, leading to a decrease in free concentration. Uptake from tissue was even faster for both compounds, with a larger decrease in free concentration due to binding to proteins and membranes. Why uptake is faster in the unstirred agarose gel and tissue systems is unclear. As sorption affinity is lower in these systems, uptake may just be faster as less mass transfer is needed to reach equilibrium. However, as binding of the compounds occurs both in agarose gel and tissue, facilitated transport might occur, leading to increased uptake rates of the compounds to the SPME fiber (Chapter 6).

The used pork muscle tissue was loaded with the test compounds by placing it in a solution spiked with the compound for 24 hours. This loading time was found to be sufficient to reach equilibrium between muscle, liver and fat tissue of rats and the organophosphate parathion *(Artola-Garicano et al. 2000)*. Based on calculations, we found that the concentrations in tissue and solution had not reached equilibrium in our experiments (Chapter 6). This means that the distribution of the compounds in the tissue is not homogeneous. However, we still found a good linear relationship between fiber concentrations and tissue concentrations, after a pre-equilibrium fiber exposure.

In general, the proposed SPME method already seems feasible as a qualitative tool in tissue for the identification of pharmaceuticals and other toxicants. However, correct calibration is necessary to relate fiber concentrations to tissue concentrations and make quantitative measurements possible.

Matrix effects

Matrix effects that can occur in samples containing protein include fouling and facilitated transport. Fouling is the binding of proteins to the fiber, and can have two effects on SPME sampling. Either the protein attached to the fiber occupies sorption sites on the fiber, making these unavailable for the analyte. In this case, uptake kinetics of the analyte will be slower. The other fouling effect is when the attached protein contains bound analyte, thereby increasing the

total amount of analyte sorbed to the fiber. This leads to an overestimation of the actual free concentration. Another matrix effect that can occur is facilitated transport. Here, depletion of free analyte close to the fiber leads to the release of bound analyte, thereby leading to an increase in uptake kinetics. The effect of fouling and facilitated transport are visualized in figure 4.

Fouling

Although fouling can have two (opposite) effects, both are caused by the binding of the matrix to the fiber. For the C18/SCX fibers, the binding of bovine serum albumin (BSA) was quantified using a Lowry assay (Chapter 5). We found a clear fouling effect, as BSA had a sorption affinity (log D_{fw}) of 0.3. This is consistent with the fouling effect for PDMS *(Oomen et al. 2000)* and polyacrylate fibers *(Heringa et al. 2006)*. Although the C18/SCX fiber contains ion-exchange sites, even higher sorption affinities for BSA were found for ion-exchange membranes (log D_{fw} = 1.8-2.0) *(Oemisch et al. 2014)*. To characterize the potential effect of the binding of BSA on the outcome of our experimental work, uptake curves for amitriptyline, amphetamine, diazepam and tramadol to the C18/SCX fiber were made with and without BSA (Chapter 5). This showed a slight decrease in uptake kinetics for the cationic compounds, and a small increase for diazepam. Therefore, we employed equilibrium sampling in all experimental work to calculate binding affinities, as changed uptake kinetics have no effect on the fiber concentration at equilibrium. However, fouling can also add compound to the fiber through the binding of occupied protein, thereby leading to an incorrect estimation of the free concentration. Using the results of the Lowry assay, we were able to quantify the potential fouling effect and how this affects the calculated binding affinities (Chapter 5). We found a maximum variation of 0.02 log units in calculated K_{BSA} values for all compounds. Recently, a mathematical model was introduced to predict the influence of protein on a compound's uptake kinetics to SPME fibers *(Alam et al. 2015)*. They found that fouling does not hinder the initial fast extraction phase, that is dominated by the free concentration in the boundary layer, but does hinder extraction in a later stage of the uptake. This is usually caused by a slow dissociation of the compound from the binding matrix, and is thus dependent on the binding affinity of the compound *(Alam et al. 2015)*. We found the largest decrease in uptake kinetics in the presence of BSA for amitriptyline, which is the compound with the highest binding affinity of the compounds tested (Chapter 5). Although this mathematical model is promising, it provides no general predictions for cationic compounds or SPME fibers employing cation-exchange.

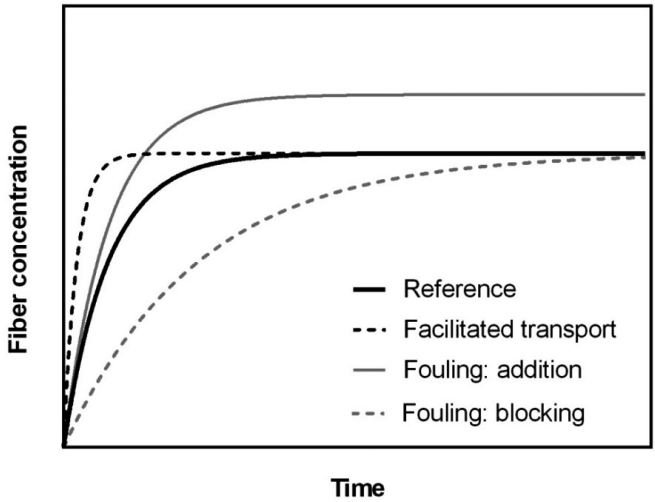

Figure 4. Influence of matrix effects on SPME uptake curves. Compared to a reference uptake curve (solid black line), either facilitated transport (dashed black line) or fouling (grey lines) can occur. Fouling can be split into an additive effect (solid grey line) or a decrease in uptake kinetics (dashed grey line). Adapted from (*Heringa & Hermens 2003*).

Facilitated transport

Facilitated transport is a diffusion layer effect, as the diffusion rate of the compound to the fiber is increased by a net desorption of analyte from the binding matrix in the stagnant water layer surrounding the fiber *(Heringa & Hermens 2003)*. Previous research found that the facilitated transport rate was increased with higher binding affinities and with an increased concentration of binding matrix *(Kramer et al. 2007)*. In our work, we saw no increase in uptake kinetics for cationic compounds from BSA solutions (Chapter 5). However, there was a clear increase in uptake kinetics for diazepam and amitriptyline from agarose gel and pork muscle tissue (Chapter 6). In general, the tortuosity in a semisolid matrix would limit the diffusion rate of the compound to the fiber. The reason for an increase in uptake into agarose gel and tissue is unclear, but it could be related to facilitated transport. Again, the mathematical model of *(Alam et al. 2015)* showed that facilitated transport occurs mainly with higher binding affinities and with higher analyte to BSA ratios. However, their research does not include complex samples such as tissue or blood that require a multicomponent approach.

In summary

The proposed method using C18/SCX SPME fibers can be easily applied in biological samples, including blood and tissue. Although correct application requires a good knowledge on uptake kinetics, potential fouling effects and correct calibration, it remains a promising tool to study postmortem redistribution. In the following section, the potential of this method to study postmortem redistribution is discussed.

POTENTIAL OF SPME TO STUDY POSTMORTEM REDISTRIBUTION

The initial scope of this research was to study postmortem redistribution. Although SPME has not yet been applied in postmortem redistribution studies, this thesis contains valuable information on distribution processes of cationic compounds and the interpretation of sampling data for ionizable compounds. Insight into these processes is essential in future applications of this SPME method. This section proposes how solid-phase microextraction can be used as a tool in forensic toxicology, including the study of postmortem drug redistribution. The discussion is focused on the practical application of SPME fibers. From an experimental point of view, it is good to realize that forensic samples always require some form of work-up which make the process time-consuming, as discussed briefly in Chapter 1. Although sample work-up is overcome by using SPME as fiber extracts are relatively clean samples, experimental work using SPME can also be time-consuming.

SPME to obtain HB:FB ratios

Heart blood to femoral blood concentration ratios (HB:FB ratios) are the most commonly used denominator of postmortem redistribution (Chapter 1). As mentioned before, the major downfall of HB:FB ratios is that the postmortem interval is usually not incorporated in this ratio. However, reference values for HB:FB ratios are widely available, as for instance published by *(Han et al. 2012)*. SPME can also be used to obtain HB:FB ratios. Both heart blood and femoral blood can be sampled simultaneously using SPME. In this case, fiber calibration to correlate fiber concentrations to blood concentrations would not even be necessary, as the ratio between the fiber concentrations should be equal to the HB:FB ratio. Short exposures of e.g. 15 minutes would be sufficient to allow for good sensitivity for most drugs (Chapter 6), although it should be noted that the co-extraction of endogenous or putrefactive compounds could decrease sensitivity *(Butzbach 2010)*. The key benefit of using SPME is the

ability to perform repeated sampling in the same body. Using this approach, the change in HB:FB ratios over time could be examined. This could help to improve developed QSAR models *(Giaginis et al. 2009, Giaginis et al. 2014)* to predict postmortem redistribution and define physicochemical properties that influence this phenomenon.

Studying postmortem redistribution including a time component

In an attempt to include the postmortem interval when studying postmortem redistribution, two studies incorporated the time between mortuary admission and postmortem examination as a measure of the postmortem time interval. The first study used a set of 30 different compounds with an average time interval between admission and autopsy of 64 hours *(Gerostamoulos et al. 2012)*. Of the compounds studied, only seven showed a more than 20% increase in blood concentrations, irrespective of the postmortem interval. However, the actual postmortem interval might be much longer than the time interval between admission and autopsy, and it is very likely that redistribution already occurred before the first sample is taken at mortuary admission. The second study tried to eliminate some of this insecurity, by including only cases where mortuary admission occurred within 24 hours after death *(Saar et al. 2012)*. They examined ten basic compounds, of which seven showed a more than 40% change in concentration, again irrespective of the postmortem interval. This indicates that postmortem redistribution occurs relatively fast (within 24 hours) after death, and shows the importance of early postmortem sampling. As both studies use different postmortem cases for different time intervals studied, they show large variations in concentrations due to interindividual differences. These studies are also limited in the number of samples that can be taken from the same body, as this might disturb the existing equilibrium. Again, using SPME repeated sampling is possible, thereby greatly enhancing the amount of data that can be obtained from a single case.

A recent study employed the use of computed tomography (CT)-guided biopsy sampling to study time-dependent postmortem redistribution *(Staeheli et al. 2015)*. In a proof of concept, they used CT imaging before autopsy to place introducer needles into the right heart ventricle, lung, liver, kidney, spleen, subcutaneous fat, muscle tissue, femoral vein and dural sac. After verification of the position of the needles, blood could be drawn using syringes, and tissue was collected using biopsy needles. With the use of CT imaging, it is possible to collect tissue samples from precise collection sites without opening of the

body. The next day, autopsy was performed, collecting samples from the same locations as had been biopsied before. Although promising, a drawback of this method is the high level of inhomogeneity between the tissue samples taking at different time points. Although biopsy samples were taken very close together, inhomogeneous tissue distribution led to high variation in data. To overcome this, the authors suggest to do a triplicate measurement at each location, resulting in at least 27 samples per case per time point investigated. The number of samples needed and the use of CT imaging make this a very time-consuming method. Another drawback is that sample collection can only start after mortuary admission. This limits sample collection during the first few hours after death, when postmortem redistribution mainly occurs. To investigate early postmortem redistribution, animal studies would still be necessary (which will be discussed further in the following section). Finally, by removing material (blood or tissue) from the body, there is a possible shift in the existing equilibrium. As reported extensively in this thesis, a change in equilibrium will refute the use of repeated sampling. However, this sampling strategy based on CT-guided biopsies does provide a novel way to study time-dependent postmortem redistribution in humans and should be explored further.

Use of test animals to study postmortem redistribution

Animal studies are to this day used to study time dependent effects of postmortem redistribution. Animals used include rats *(Hilberg et al. 1992)*, rabbits *(Pélissier-Alicot et al. 2006)*, pigs *(Brunet et al. 2010)* and dogs *(Johnson et al. 2015)*. The choice of animal species is in part dependent on the compound studied. For instance, the investigation of three beta-blockers requires animals with a gall bladder that produce bile, as these compounds easily enter the enterohepatic cycle *(Pélissier-Alicot et al. 2006)*. However, it would seem most important that an animal has a large likeness to humans, both in physiology as in size, which is why pigs are often used as a surrogate *(Brunet et al. 2010)*. Postmortem redistribution most likely occurs faster in smaller animals, as passive diffusion seems to be the dominant transport mechanism. Although postmortem redistribution studies using animals can provide detailed information on tissue distribution and average redistribution processes, they suffer from large variations in determined concentrations as different animals have to be used at each time point. It is very likely that interindividual differences occur in any biological specimen, including humans. Eliminating or at least reducing this seems essential when studying changing concentrations in any system. Repeated sampling using SPME would allow the sampling of a

single animal (or human) at each time point, thereby gaining insight into the true process of postmortem redistribution.

Quantitative measurements in postmortem samples

When using SPME as a quantitative tool in complex samples, sensitivity can be decreased due to the co-extraction of endogenous compounds. As shown in Chapter 3, competition effects for the C18/SCX fiber occur above 10 mmol/L of total fiber loading. In complex samples, sorption is not limited to the parent drug, but also includes its metabolites and endogenous compounds. It can be argued that metabolites usually are more hydrophilic than the parent compound, and will thus have a lower sorption affinity and consequently fiber concentration. For endogenous compounds, which include fatty acids, thyroxine and bilirubin, typical concentrations *in vivo* are usually lower than those of exogenous compounds, so their contribution to total fiber loading will be lower. Also, endogenous compounds are usually highly protein-bound, thereby limiting the free concentration available for uptake to the fiber. However, competition might be mostly expected from certain widely used consumer products, such as coffee and cigarettes. Caffeine concentrations reach the mg/L range after an average dose of 120 mg *(Baselt 2014)* which is in the same order of magnitude as therapeutic, toxic and lethal drug concentrations. Although plasma concentrations of nicotine are substantially lower than those of caffeine, it is a cationic compound (pKa 8.9, *(ChemAxon, www.chemicalize.org)*) with significant sorption to the C18/SCX fiber (log D_{fw} 2.4, Chapter 4). So especially for high affinity analytes and high concentration analytes, competition effects are expected to occur. Above a total fiber loading of 10 mmol/L, sorption to the fiber becomes strongly nonlinear which leads to calibration problems for quantitative measurements. One solution would be to use shorter sampling times, which can be performed easily without loss of sensitivity as shown in Chapter 3 and Chapter 5. Another option is to perform a total organic carbon analysis on the fiber extract using GC-FID (flame ionization detection). This could provide an estimation of the total fiber concentration. However, the most convenient solution might be to use SPME fibers based on a bulk polymer such as PDMS, as these probably show no competition effects. The downside of these type of fibers is the lack of sensitivity for ionized compounds, but neutral compounds (such as those routinely screened during postmortem examination, see table 1, Chapter 1) could be captured effectively.

Other potential future forensic applications of SPME

Besides studying postmortem redistribution, SPME could also prove beneficial in other forensic applications.

On site postmortem sampling

An interesting feature of SPME is the size of the fibers. The used fibers are very small, and are currently also biocompatible because of to the use of surgical materials as the fiber support. A potential application of SPME could be on site sampling, by incorporating SPME fibers as part of the tool kit of crime scene investigators. On site sampling could be limited to easily accessible bodily fluids, such as vitreous humor, although placing the fiber directly in a vein (e.g. cephalic vein or jugular vein) should be possible with some training. Quick screening of these first fiber extracts could give an indication of the compounds present, thereby reducing analysis time on further samples and providing useful information before autopsy. Further analysis of bodily fluids or tissue homogenates can then be customized to the analytes detected during this first screening. When drugs are detected during this first screening, this would then warrant further sampling to study postmortem redistribution effects after mortuary admission until the definitive autopsy.

Doping

Competitive sports are often linked to doping, leading to stringent monitoring of drugs in sports by for instance the World Anti-Doping Agency (WADA). Currently, athletes are subject to blood draws and urine checks. SPME could be applied directly *in vivo*, by placing the fiber in the athlete's vein for a short amount of time. Research has showed that the C18/SCX fibers are capable of extracting even very polar drug metabolites, up to log K_{ow} values of -7 *(Vuckovic & Pawliszyn 2011)*. Even if *in vivo* sampling is not feasible, *ex vivo* sampling using SPME could already provide several benefits. First of all, drugs extracted to the SPME coating are no longer subject to degradation or metabolism. This means that storage conditions of bodily fluids during transportation to the lab (now as fiber extracts) can become more flexible. Another benefit is the potential for an automated high throughput system, where samples can be analyzed without prior work-up and limited handling. Reportedly, WADA is already participating in such a project *(Bojko & Pawliszyn 2013)*.

Other applications

SPME could also be used in a number of other forensic applications. These include environmental forensics, which is the sampling of waste water to detect drug residues, or the illegal discharging of drugs or its raw materials *(Kabir et al. 2013)*. Recent research has focused on custom-made SPME samplers for *in vivo* sampling of compounds in human saliva *(Bessonneau et al. 2015)* and the use of plastic as a fiber support to reduce costs and increase robustness and user friendliness *(Reyes-Garcés et al. 2015)*.

In summary

SPME could be incorporated into currently used methods to detect the presence of postmortem redistribution, e.g. by calculating HB:FB ratios. Moreover, this tool could be especially useful when studying the time component of postmortem redistribution. Although the method seems promising, there are still some factors that need to be further investigated. These include the effects of co-extracted compounds such as caffeine and nicotine.

CONCLUDING REMARKS AND PRACTICAL IMPLICATIONS

This thesis is the result of a project that aimed to develop new analytical techniques to study postmortem drug redistribution. A sampling method based on solid-phase microextraction (SPME) was investigated and optimized for a number of pharmaceutical and illicit drugs. The extraction mechanism of the C18/SCX fiber is extensively researched and the method was able to extract compounds from complex matrices including whole blood and tissue. Although no true forensic cases were investigated during this project, SPME is a relatively new sampling technique which is constantly evolving. It therefore remains a highly promising tool in forensic toxicology.

Interpretation of postmortem found drug concentrations can be difficult due to postmortem redistribution phenomena. Insight into postmortem drug redistribution is lacking as the amount of sample is limited and repeated sampling is impossible with current techniques.

- Sampling methods based on solid-phase microextraction allow for repeated sampling in the same matrix. Specifically, the C18/SCX fiber has a large chemical applicability domain that includes cationic, neutral and anionic pharmaceuticals and illicit drugs.
- The C18/SCX fiber can be used as direct sampling tool in biological fluids to obtain accurate quantitative drug concentrations. However, matrix effects such as fouling and facilitated transport influencing the uptake of drugs to the fiber should always be considered.
- Direct postmortem application of the C18/SCX fiber is feasible, but competition effects are to be expected both from endogenous compounds and widely used consumer products. The time interval of fiber exposure should therefore be chosen carefully, long enough to obtain good sensitivity, yet short enough to minimize competition effects.
- Although qualitative measurements in tissues could be easily obtained using the C18/SCX fiber, more sorption data on a wide set of compounds and from a variety of tissue types is needed to quantitatively correlate fiber concentrations with tissue concentrations.
- Future pilot experiments could focus on series of systematic “fast” SPME (biocompatible C18 or C18/SCX) sampling alongside tissue sampling in drug suspected forensic cases, to create more feeling for quantitative feasibility with specific pharmaceuticals and illicit drugs.

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SUMMARIES

BACKGROUND

Forensic toxicology is a discipline in toxicology that aids in the legal investigation of death, poisoning or drug use. A small, yet complex part of forensic toxicology is postmortem toxicology, which aims to interpret toxicological findings after death. An important factor complicating this interpretation is postmortem drug redistribution. As active processes that drive drug concentration gradients stop postmortem, passive diffusion will cause artefactual changes in blood concentrations. Many compounds show some level of postmortem redistribution, but especially basic drugs show high levels of postmortem redistribution, due to a postmortem decrease in body pH.

To be able to study kinetic processes, such as postmortem drug redistribution, it is essential to sample drug concentrations at several time points. Limitations in forensic studies include the amount of available sample and sampling can be constrained from an ethical or legal point of view. The most important limitation, however, is that by removing blood or tissue, the existing equilibrium is disturbed, thereby changing diffusion processes and making further sampling not representative of the true process of postmortem redistribution. To overcome this problem, specific sampling techniques can be applied that leave the sample undisturbed. Solid-phase microextraction (SPME) represents such a sampling technique. With SPME, an equilibrium extraction is obtained, making repeated sampling possible in certain conditions such as negligible depletion. Another important feature of SPME is that only the free concentration partitions towards the fiber.

In this thesis, a prototype SPME fiber is described, which utilizes two mechanisms of extraction. The fiber is coated with C18 chains and propylsulfonic acid, which act as strong cation exchange groups. This dual extraction mechanism gives the fiber its name: mixed-mode fiber or C18/SCX fiber. The C18/SCX fiber is especially efficient at sorbing relatively hydrophobic, cationic compounds (such as basic drugs).

AIM

The main aim of this thesis is the development and validation of SPME as a potential sampling tool in forensic applications. The focus is on the sampling of ionized pharmaceuticals and illicit drugs using the C18/SCX fiber. Whereas the partition behavior and sampling methodology for neutral toxicants is reasonably well documented, understanding the distribution processes of ionizable compounds and interpreting sampling data poses various scientific challenges.

EXPERIMENTAL WORK

The experimental work in this thesis is divided into two parts: the investigation of the sorption mechanism of cationic compounds to the C18/SCX fiber (Chapter 2-4) and the application of the C18/SCX fiber to determine freely dissolved concentrations and study sorption processes (Chapter 5 & 6).

The C18/SCX fiber was investigated as novel sampling coating in Chapter 2 using amphetamine as a model compound. Sorption of amphetamine to the C18/SCX fiber was compared to existing SPME fibers with polyacrylate (PA) or C18 coating. This showed that the affinity of amphetamine for the C18/SCX fiber was 20 to 180 times greater at pH 7.4 than for C18 or PA fibers, respectively. The enhanced affinity was attributed to the ion-exchange phase in the coating. However, neutral amphetamine (pH 11) also showed a higher affinity to the C18/SCX fiber compared to PA. Due to the limited presence of ion-exchange groups in the coating, sorption isotherms level off at higher tested aqueous concentrations. This also explains observed competition effects by inorganic cations, which compete for the SCX sites and decreased sorption of amphetamine.

To further elucidate the sorption mechanism of the C18/SCX fiber, Chapter 3 focused on both amphetamine and the more hydrophobic cation amitriptyline. Due to this higher hydrophobicity, sorption affinity of amitriptyline to the C18/SCX fiber is markedly higher than that of amphetamine. A direct comparison between the C18/SCX fiber and C18 fiber over a wide pH range for the cationic compounds amitriptyline, amphetamine and trimethoprim showed an increased sorption affinity for the C18/SCX fiber for all compounds, although sorption affinities for the C18 fiber were only less than 1 log unit lower. This indicates that the SCX groups have an additional effect on the sorption

of cationic compounds, but this effect is relatively small. Also the role of negatively charged silanol groups in the C18 and C18/SCX fiber seems small, as anionic diclofenac sorbed strongly to the C18 fiber. Competition effects described previously were investigated further by using binary mixtures of amphetamine and amitriptyline. This showed that competition occurs when the total fiber loading exceeds 10 mmol/L coating.

In Chapter 4, sorption data on 31 structurally diverse compounds was combined to extend the data set and to investigate the effects of chemical structure on sorption affinity. The data set included 27 cations, three neutral compounds and one anionic compound. The affinity of the tested organic cations without H-bond forming capacity increased with longer alkyl chains, and decreased with increased branching and presence of multiple aromatic rings (at an equal number of carbon atoms). For these compounds, a clear relationship was found between sorption affinity and log K_{ow} of the neutral form of the compound. Cations with H-bond capacity sorbed stronger than based on their log K_{ow}, as did the neutral compounds. Results for anionic diclofenac indicate that the C18/SCX fiber might also be useful for sampling of organic anions.

Starting in Chapter 5, the application of C18/SCX-SPME as sampling tool for freely dissolved concentrations is studied. Proteins (bovine and human serum albumin) were added to solutions containing amphetamine, amitriptyline, diazepam or tramadol. Binding affinities calculated from measured free concentrations were very similar to reported literature values. The influence of fouling (binding of protein to the SPME coating) was quantified and its effect on calculated binding affinities was determined. C18/SCX fibers were then directly exposed to spiked plasma and whole blood samples. Measured bound fractions in plasma were comparable to literature reference values. Bound fractions in whole blood were always higher than in plasma, due to binding of the compound to cell membranes. The C18/SCX fiber might especially be beneficial for the sampling of highly protein-bound cations, as sampling the small neutral fraction of these compounds using PA or polydimethylsiloxane (PDMS) coatings might not reach the sensitivity required for quantification.

The use of C18/SCX-SPME as a sampling tool in semi-solid tissues was investigated in Chapter 6. Firstly, agarose gel was used as a semi-solid tissue surrogate, to mimic changes in matrix tortuosity and hindered diffusion as expected to occur in tissue. Sorption of diazepam and amitriptyline from

agarose was well above detection limits, even when using short sampling times. The tissue used was pork muscle, which was loaded with the analytes using a 24h incubation in spiked phosphate-buffered saline (PBS). Again, detectable fiber concentration were found after direct sampling in the loaded tissue, including short sampling times and different loading concentrations in tissue. Uptake of the analytes from agarose gel and tissue to the C18/SCX fiber was faster than from PBS (a liquid matrix), indicating that mass transfer might be faster in these models. Although more work is needed to elucidate this phenomenon, these preliminary results already show that the C18/SCX fiber is a sensitive tool to determine tissue concentrations of neutral and cationic compounds.

CONCLUSIONS

The experimental work in Chapter 2-4 has shown the benefit of the C18/SCX fiber for the sorption of cationic compounds. This fiber can be used to measure freely dissolved concentration and with that understand distribution processes for cationic compounds. Unlike the partitioning of neutral compounds, the distribution of cationic compounds is still a relatively little studied topic. Their distribution *in vivo* seems to be governed by different proteins and lipids compared to neutral compounds. Using partitioning data on different binding matrices, a simple model can be constructed to predict distribution *in vivo*. Improved knowledge on this distribution will aid in assessing the bioaccumulation potential of cationic compounds. SPME can be useful to measure freely dissolved concentrations, and with that calculate sorption and assess distribution processes. In Chapter 5 & 6, the C18/SCX fiber is used to study protein binding and uptake from tissue. The C18/SCX fiber was furthermore shown to perform well in extracting basic drugs from semi-solid tissues, which is an essential step towards the application of SPME-based methods in forensic toxicology. This proves the benefit of SPME in aqueous and semi-solid complex samples, as the method is simple and it provided good quantitative results. The C18/SCX fiber especially provided a good sensitivity for basic pharmaceuticals, as it is capable of extracting the charged species. For SPME to be applied in forensic studies, some difficulties will need to be addressed both in the implementation of the method (e.g. fast postmortem sampling) and in obtaining good quantitative results (e.g. competition effects).

SAMENVATTING VOOR NIET-INGEWIJDEN

CONTEXT

Een belangrijk onderdeel van de forensische (gerechtelijke) toxicologie is het opsporen van toxische verbindingen zoals geneesmiddelen en drugs en het beoordelen van postmortaal (na het overlijden) gevonden bloedwaarden van geneesmiddelen en drugs (geneesmiddelconcentraties). De hoogte van deze geneesmiddelconcentraties kan aantonen of een bepaald geneesmiddel of drug heeft bijgedragen aan het overlijden of zelfs de dood heeft veroorzaakt. Door verschillende veranderingen die na het overlijden plaatsvinden in het lichaam kunnen deze concentraties echter veranderen. Deze veranderingen in concentraties noemt men postmortale herverdeling. Dit proces is moeilijk in kaart te brengen omdat herhaaldelijk bloed afnemen niet mogelijk is zonder de postmortale herverdeling te beïnvloeden. Voornamelijk verbindingen die positief geladen zijn (kationen) ondergaan postmortale herverdeling. Daar komt bij dat de meeste geneesmiddelen en drugs positief geladen zijn bij de pH (zuurgraad) van het lichaam. Uit literatuur weten we veel over het gedrag van neutrale verbindingen in het lichaam en hoe we deze kunnen meten. Echter, over het gedrag van geladen verbindingen is veel minder bekend en de gebruikelijke meetmethodes zijn moeilijk toe te passen voor geladen verbindingen.

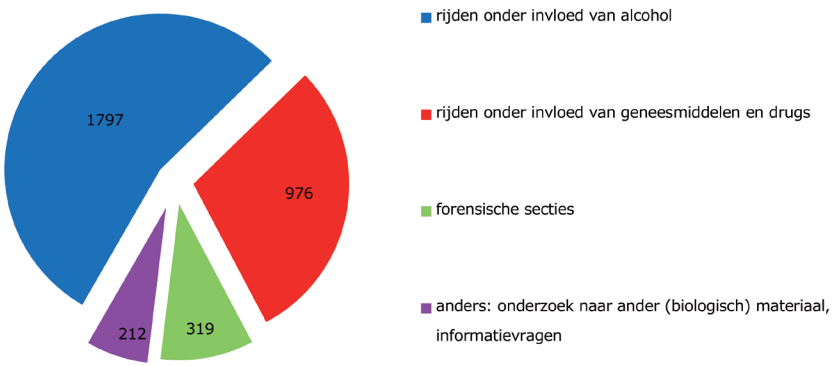
DOEL

Een belangrijk doel van dit proefschrift is om nieuwe meetmethoden te ontwikkelen waarmee postmortale herverdeling in kaart kan worden gebracht. Hiervoor is een bemonsteringstechniek onderzocht op basis van solid-phase microextraction (SPME), die in het bijzonder gericht is op geneesmiddelen en drugs die positief geladen zijn (kationen).

ACHTERGROND

Forensische toxicologie

De forensische toxicologie omvat al het toxicologisch onderzoek in gerechtelijke zaken. Dit kan onderzoek zijn naar een doodsoorzaak, een vergiftiging of drugsgebruik. In Nederland wordt dit onderzoek uitgevoerd door het Nederlands Forensisch Instituut (NFI). Figuur 1 laat zien dat het NFI meer dan 3000 zaken heeft onderzocht in 2014. Slechts een klein deel daarvan zijn zaken waar forensische secties worden verricht. Dit specifieke deel van de forensische toxicologie is echter zeer complex en vergt dus meer tijd dan



Figuur 1. Overzicht van alle zaken binnen forensische toxicologie in 2014, zoals gerapporteerd door het Nederlands Forensisch Instituut.

andere zaken. Een belangrijke oorzaak hiervan is postmortale herverdeling, die de interpretatie van postmortale geneesmiddel- en drugconcentraties bemoeilijkt.

Als iemand overlijdt, veranderen of stoppen de normale processen in het lichaam. Doordat het hart niet meer pompt, het bloed niet meer door het lichaam stroomt en er geen ademhaling plaatsvindt, krijgt het lichaam geen zuurstof meer. Zonder zuurstof wordt glucose omgezet in melkzuur, waardoor het lichaam veel zuurder wordt dan normaal. Zuurstof is ook nodig voor de aanmaak van ATP (adenosine trifosfaat), de energiestof van het lichaam. ATP zorgt normaal gesproken voor een specifieke balans tussen natrium en kalium ionen in alle cellen van het lichaam. Zonder ATP komt er teveel natrium in de cellen. In een poging dit te compenseren, stroomt er veel water de cellen in. Hierdoor kunnen de cellen barsten. De zuurgraad van het lichaam zorgt er nog extra voor dat cellen kapot gaan doordat er enzymen vrij komen die de celwand beginnen af te breken.

Geneesmiddelen en drugs worden tijdens het leven voornamelijk opgeslagen in bepaalde organen (lever, longen, darmen). Postmortale herverdeling is in principe de diffusie (verplaatsing) van geneesmiddel van een plek in het lichaam met een hoge concentratie (meestal vanuit de organen) naar een plek met een lage concentratie (meestal richting het bloed). Hierdoor kunnen

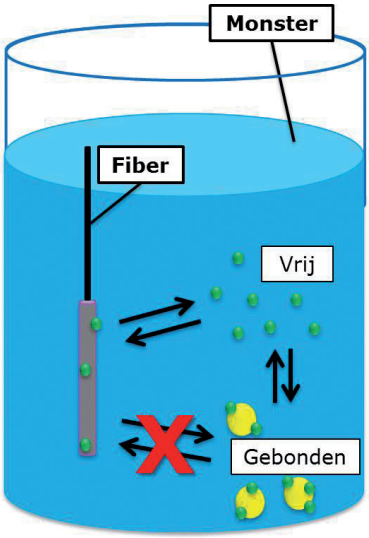
gemeten bloedconcentraties van geneesmiddelen en drugs dus postmortaal (veel) hoger uitvallen dan dat ze op het moment van overlijden waren. Er zijn echter veel factoren die meespelen in de mate waarin postmortale herverdeling plaatsvindt. Dit zijn lichamelijke factoren, zoals hoe lang iemand al dood is en in welke staat van ontbinding het lichaam zich bevindt, maar ook de eigenschappen van het geneesmiddel spelen mee, in het bijzonder of een geneesmiddel positief geladen is.

Om postmortale herverdeling in kaart te brengen, is het noodzakelijk om op verschillende momenten na de dood te bepalen hoe hoog de geneesmiddelconcentraties in het bloed en in de weefsels zijn. Dit kan niet door bloed of weefsel uit het lichaam te nemen, want dan wordt het proces dat wordt bestudeerd teveel verstoord. Om dit soort onderzoek uit te voeren, is een snelle en gevoelige bemonsteringstechniek nodig, die zowel in bloed als weefsel kan worden toegepast maar die zelf niet de concentratie en de bestaande processen verstoord. In dit proefschrift wordt daarvoor solid-phase microextraction gebruikt.

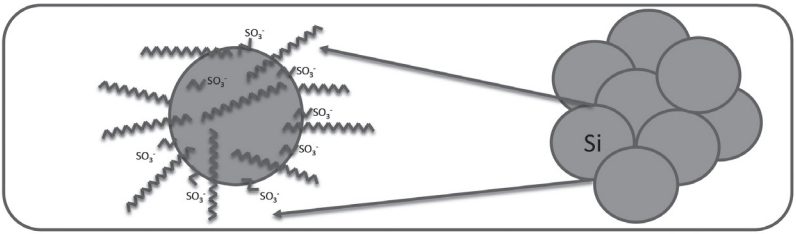
Solid-phase microextraction

Solid-phase microextraction (SPME) is een bemonsteringstechniek gebaseerd op extractie. Een SPME fiber bestaat uit een glazen of metalen kern waarop een polymeer is aangebracht. Er bestaan verschillende van deze polymeren of coatings, die in staat zijn om geneesmiddelen of andere verbindingen in zich op te nemen (dit noemen we sorptie). In vergelijking met andere extractietechnieken komt er bij SPME uiteindelijk slechts een deel van het in het monster aanwezige geneesmiddel in de fiber terecht. De hoeveelheid geneesmiddel die wordt opgenomen door de fiber is in bepaalde gevallen zo klein, dat de oorspronkelijke geneesmiddelconcentratie nauwelijks wordt veranderd. Hierdoor is het mogelijk om met SPME meerdere malen achter elkaar te meten, zonder dat concentraties en postmortale processen worden verstoord. Een ander belangrijk onderdeel van SPME is dat wanneer een geneesmiddel deels wordt gebonden door eiwitten (zoals in bloed vaak gebeurt), alleen de vrije concentratie (dit is de concentratie van het geneesmiddel/drug dat niet gebonden is aan onder andere eiwitten) wordt opgenomen door de fiber (figuur 2).

De eerste toepassingen van SPME fibers waren alleen geschikt voor neutrale verbindingen. In dit proefschrift ligt de nadruk echter op geladen verbindingen, en in het bijzonder op positief geladen geneesmiddelen en drugs. Of een stof



Figuur 2. Bij blootstelling van een SPME fiber aan een monster met een bepaalde geneesmiddelconcentratie ontstaat er evenwicht tussen de vrije concentratie en de geneesmiddelconcentratie op de fiber. Stoffen die gebonden zijn aan bijvoorbeeld eiwitten worden niet opgenomen door de fiber.



Figuur 3. Schematische weergave van de coating van de C18/SCX fiber. Lange C18 ketens en propylsulfonzuur (SO_3-, *strong cation exchange* of SCX groepen zijn gebonden aan silica deeltjes. Deze silica deeltjes worden vervolgens via een speciale lijm vastgemaakt aan een metalen kern. De C18/SCX fibers zijn prototype fibers van Supelco.

geladen is, is afhankelijk van de pH (zuurgraad) van het monster en van de pKa (zuurconstante) van de stof. Als de pH van het monster gelijk is aan de pKa van de stof, dan is de helft van de aanwezige moleculen geladen en de andere helft niet geladen. Een base raakt steeds verder positief geladen naarmate de pH daalt onder de pKa. Voor een zuur geldt het tegenovergestelde, naarmate de pH stijgt boven de pKa raakt een zuur steeds verder negatief geladen.

Door middel van nieuw ontwikkelde coatings is het nu mogelijk om geladen verbindingen te meten met behulp van SPME. In dit proefschrift wordt gebruik gemaakt van de zogeheten mixed-mode of C18/SCX fiber. Deze bevat een coating die bestaat uit twee delen. De ene fase bestaat uit lange C18 ketens waardoor lipofiele (vet-oplosbare of door vet aangetrokken) verbindingen goed te vangen zijn. De andere fase bestaat uit sterk negatief geladen groepen, in dit geval propylsulfonzuur (ook wel *strong cation exchange* (SCX) genoemd en vertaald "sterke uitwisseling met kationen"). Hierdoor is deze fiber uitermate geschikt voor positief geladen verbindingen. Figuur 3 geeft een schematische weergave van de coating van de C18/SCX fiber.

EXPERIMENTEEL WERK

Het experimentele werk in dit proefschrift bestaat uit twee delen: het onderzoek naar het sorptiemechanisme van kationen naar de C18/SCX fiber (Hoofdstuk 2-4) en de toepassing van de C18/SCX fiber om vrije concentraties van geneesmiddelen en drugs te bepalen en sorptieprocessen te bestuderen (Hoofdstuk 5 & 6).

Hoofdstuk 2

In dit hoofdstuk wordt het eerste gebruik van de C18/SCX fiber beschreven. De sorptie van amfetamine naar deze fiber en twee andere fibers (polyacrylaat fibers en C18 fibers) wordt vergeleken. Hieruit blijkt dat de sorptie affiniteit van positief geladen amfetamine (bij fysiologische pH 7.4) voor de C18/SCX fiber vele malen hoger is dan voor de andere SPME fibers. Dit lijkt te komen doordat de C18/SCX fiber negatief geladen groepen bevat en dus de positief geladen amfetamine goed kan vangen, terwijl de andere SPME fibers deze negatief geladen groepen niet hebben. Ook hebben we de sorptie affiniteit van neutraal amfetamine (bij pH 11) vergeleken. Hier bleek de C18/SCX fiber het ook beter te doen dan polyacrylaat fibers. Hieruit kan geconcludeerd worden dat met de C18/SCX fiber gevoeliger kan worden gemeten bij verschillende pH's. Een nadeel van de C18/SCX fiber is dat de negatief geladen groepen maar

beperkt aanwezig zijn. Bij hogere concentraties van amfetamine beginnen deze groepen bezet te raken, waardoor vanaf dat punt minder amfetamine door de fiber kan worden opgenomen dan bij lagere concentraties. Ook kunnen geladen ionen (zoals natrium en kalium) in het monster aan deze groepen binden. Hierdoor wordt de sorptie van amfetamine minder omdat de negatief geladen groepen bezet zijn (competitie).

Hoofdstuk 3

Om nog meer inzicht te krijgen in het sorptiemechanisme van de C18/SCX fiber wordt in dit hoofdstuk het onderzoek naar amfetamine uitgebreid met een ander kationisch geneesmiddel, namelijk amitriptyline. Amitriptyline is hydrofober dan amfetamine, wat betekent dat deze minder van water houdt. Hierdoor heeft amitriptyline een hogere sorptie affiniteit naar de fiber dan amfetamine. De sorptie affiniteit van amitriptyline, amfetamine en een derde kation (trimethoprim) voor de C18/SCX fiber werd vergeleken met de C18 fiber. Hieruit bleek dat de drie kationen allemaal een hogere affiniteit hebben voor de C18/SCX fiber dan voor de C18 fiber. De affiniteit voor de C18 fiber was weliswaar lager, maar niet extreem veel lager. Hieruit kon worden geconcludeerd dat de SCX groepen in de C18/SCX fiber een bijdrage leveren aan de sorptie affiniteit van kationen maar dat dit effect niet heel groot is. Ook de competitie effecten, die gezien werden in het vorige hoofdstuk, werden verder uitgediept. De eerder gemeten sorptie affiniteit van amfetamine en amitriptyline alleen naar de C18/SCX fiber werd vergeleken met de sorptie affiniteit van mengsels van amfetamine en amitriptyline samen. Hieruit bleek dat competitie alleen optreedt bij hogere concentraties, dus wanneer de SCX groepen bezet zijn. Verder bleek dat het kation met de hoogste sorptie affiniteit (amitriptyline) het “wint” van het kation met een lagere sorptie affiniteit (amfetamine).

Hoofdstuk 4

Om te laten zien dat de C18/SCX fiber in staat is om allerlei geneesmiddelen en drugs te kunnen meten werd in dit hoofdstuk een verzameling gemaakt van alle verbindingen die tijdens dit project met deze fiber waren gemeten. Dit werd een set van 31 verschillende verbindingen, waarvan 27 kationen, drie neutrale geneesmiddelen en één anion. Door de sorptie affiniteit te vergelijken met de hydrofobiciteit (waterafstotendheid) van de verbindingen konden een paar conclusies worden getrokken. Voor de kationen was er een duidelijk verschil te zien tussen verbindingen die waterstofbruggen kunnen vormen en verbindingen die dat niet kunnen. Een waterstofbrug ontstaat wanneer twee

verschillende verbindingen zo dicht tegen elkaar aan zitten dat ze elektronen gaan delen. De verbindingen moeten dan wel bepaalde specifieke groepen bevatten, maar dit kan bijvoorbeeld gebeuren tussen een geneesmiddel of drug en de fiber coating. Voor de verbindingen die geen waterstofbruggen kunnen vormen is er een duidelijke relatie tussen sorptie affiniteit en hydrofobiciteit. Voor deze verbindingen geldt: hoe hoger de hydrofobiciteit, hoe hoger de sorptie affiniteit. Deze relatie kan gebruikt worden om de sorptie affiniteit te voorspellen. Voor kationen die wel waterstofbruggen kunnen vormen, komt deze voorspelling echter niet uit. Deze verbindingen hebben een hogere sorptie affiniteit dan wordt voorspeld op basis van hun hydrofobiciteit. Dit laat zien dat de C18/SCX fiber in staat is om verschillende soorten interacties aan te gaan met geneesmiddelen, wat uiteindelijk bijdraagt aan de gevoeligheid van deze fiber. Ook neutrale en negatief geladen verbindingen konden worden gemeten met deze fiber, wat laat zien dat deze fiber toepasbaar is voor alle soorten geneesmiddelen en drugs.

Hoofdstuk 5

Vanaf dit hoofdstuk wordt SPME als bemonsteringstechniek gebruikt om vrije concentraties van geneesmiddelen te meten. In dit hoofdstuk wordt beoordeeld of de C18/SCX fiber gevoelig genoeg is om geneesmiddelen uit complexe monsters zoals bloed te halen. Eerst werden eiwitten toegevoegd aan oplossingen van geneesmiddelen in water. Hierdoor neemt de vrije concentratie van deze geneesmiddelen af, omdat een deel ervan bindt aan de eiwitten. Met SPME kan deze vrije concentratie worden gemeten. Hieruit kan de affiniteit van het geneesmiddel voor het eiwit worden afgeleid. De geneesmiddelen die bestudeerd zijn in dit hoofdstuk waren de kationen amitriptyline, amfetamine en tramadol en de neutrale stof diazepam. De affiniteit van deze stoffen voor de geteste eiwitten komen overeen met waardes uit de literatuur, wat laat zien dat SPME een geschikte manier is om vrije concentraties te meten. Het is van belang dat het eiwit zelf niet bindt aan de SPME fiber, omdat hierdoor metingen verstoord kunnen raken. Eiwitten binden in kleine hoeveelheden aan de C18/SCX fiber, maar in de meeste gevallen leidt dit niet tot een verstoring in de metingen. Van waterige oplossingen met eiwit werd overgestapt naar complexe vloeistoffen, in dit geval plasma en bloed. Plasma is de vloeistof die overblijft als je bloed laat staan en de rode en witte bloedcellen naar beneden zijn gezakt. Het deel van de geneesmiddelen dat gebonden is aan eiwitten in het plasma kwam wederom overeen met literatuurwaardes. Dit gebonden deel is in bloed hoger dan in plasma, omdat bloed naast eiwitten ook nog rode bloedcellen bevat waar geneesmiddelen aan kunnen binden. Uit dit onderzoek blijkt dat

de C18/SCX fiber gevoelig genoeg is om vrije concentraties van geneesmiddelen te meten.

Hoofdstuk 6

Niet alleen is de toepassing van de C18/SCX fiber getest in vloeibare monsters zoals bloed. In dit hoofdstuk wordt de toepassing in meer (half-) vaste materialen zoals weefsel beschreven. In weefsel wordt verwacht dat diffusie (verplaatsing) van een geneesmiddel naar de fiber minder snel gaat. Om deze verminderde diffusie te testen is gebruik gemaakt van een gel op basis van het polymeer agarose als vervanging van echt weefsel. De stoffen amitriptyline en diazepam konden goed gemeten worden in deze agarose gel, zelfs wanneer de fiber maar kort werd blootgesteld aan deze gel. Daardoor kon er verder worden getest met echt weefsel. Hiervoor werd varkensvlees in stukjes gesneden en 24 uur in een badje met het geneesmiddel in oplossing gelegd. Een deel van het opgeloste geneesmiddel trekt zo in het weefsel. Hierna werd een SPME fiber in het stukje vlees geplaatst. Ook hier konden amitriptyline en diazepam gevoelig worden gemeten, ook na korte blootstelling en met lage concentraties in het vlees. Tegen de verwachting in werden de stoffen sneller opgenomen uit de agarose gel en het echte weefsel dan uit de vloeibare materialen. Het lijkt erop dat de verminderde diffusie een minder grote rol speelt dan gedacht. Waarom de opname uit gel en weefsel sneller is blijft nog onduidelijk, maar dit onderzoek heeft wel aangetoond dat de C18/SCX fiber kan worden gebruikt voor bepalingen van lage weefselconcentraties van geneesmiddelen en drugs.

DISCUSSIE

Het experimentele werk in dit proefschrift laat zien dat de C18/SCX fiber effectief kan worden toegepast voor kationen (Hoofdstuk 2-4). Met deze fiber kunnen vrije concentraties gevoelig worden bepaald om zo uitwisselingsprocessen van kationen te kunnen bestuderen. Dit is een relatief weinig bestudeerd onderwerp. In het menselijk lichaam zijn voor deze distributieprocessen andere eiwitten en lipiden voor kationen dan voor neutrale verbindingen. Door meer data te verzamelen kunnen modellen worden gemaakt om deze distributie te voorspellen. In Hoofdstuk 5 & 6 wordt de C18/SCX fiber ingezet om dit soort data te verzamelen. Voor een toekomstige toepassing van SPME in de forensische toxicologie moeten nog wat hordes worden genomen die zowel het gemak van de methode als de kwaliteit van de resultaten verbeteren.

CONCLUSIE

Het onderzoek in dit proefschrift geeft een gedetailleerd inzicht in het extractiemechanisme van de C18/SCX fiber. Verschillende sorptieprocessen konden effectief in kaart worden gebracht, voornamelijk voor positief geladen geneesmiddelen en drugs. De C18/SCX fiber is verder een effectieve bemonsteringstechniek in weefsels, wat een essentiële stap is naar een toekomstige toepassing van SPME binnen de forensische toxicologie.

APPENDIX

DANKWOORD

Aangezien dit het deel van het proefschrift is waar iedereen als eerste naartoe bladert, wil ik graag beginnen met iedereen bedanken die de moeite heeft gedaan om dit boekje open te slaan! Misschien vind je je naam hieronder nog ergens terug, maar in ieder geval bedankt voor de interesse in mijn proefschrift!

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CURRICULUM VITAE

Hester Peltenburg (1987) was born in Utrecht, growing up in both Utrecht and Driebergen. She attended Katholieke Scholengemeenschap De Breul in Zeist, where she graduated in 2005. She then started studying Pharmacy at Utrecht University. During her bachelor's, she wrote a thesis on sickle cell disease under the supervision of prof. dr. Koen Mertens. He encouraged her to rewrite this thesis into an article in Pharmaceutisch Weekblad (a specialist journal for Dutch pharmacists). During her master's, she performed her 6 month research internship at the department of Plasma Proteins of Sanquin Research (Amsterdam). Here, she investigated the role of the hemostatic protein von Willebrand factor on the adhesion of sickle red blood cells to vascular endothelial cells. This work was supervised by prof. dr. Koen Mertens (as head of the department and UU supervisor), dr. Jan Voorberg (as daily supervisor) and dr. Karin Fijn van Draat (as consulting specialist, pediatric hematologist at the Amsterdam Medical Centre). After successfully finishing her master's degree, she started as a PhD candidate at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University in 2012. Her PhD project was a collaboration between IRAS and the Netherlands Forensic Institute (NFI) and aimed at developing and validating a sampling method based on SPME as future novel technique in forensic applications. This project was supervised by prof. dr. Martin van den Berg (IRAS), dr. Joop Hermens (IRAS), dr. Ingrid Bosman (NFI) and dr. Steven Droge (IRAS).

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