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## DRUID

Driving under the Influence of Drugs, Alcohol and Medicines

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# Per se limits - Methods of defining cut-off values for zero tolerance

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Task 1.4 Establishment of cut-off values for *per se* legislation.

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## Contents

<b>1</b>	<b>EXECUTIVE SUMMARY</b> .....	<b>5</b>
<b>PART 1: PER SE LIMITS</b> .....		<b>7</b>
<b>2</b>	<b>INTRODUCTION</b> .....	<b>7</b>
<b>3</b>	<b>OBJECTIVES</b> .....	<b>8</b>
<b>4</b>	<b>DIFFERENT LEGAL APPROACHES</b> .....	<b>9</b>
4.1	INTRODUCTION .....	9
4.2	DEFINITION OF CUT-OFFS.....	10
<b>5</b>	<b>OVERVIEW OF EUROPEAN REGULATIONS CONCERNING PER SE LEGISLATION</b> .....	<b>12</b>
5.1	DISCUSSING DIFFERENT REGULATIONS IN NORWAY, THE NETHERLANDS AND DENMARK .....	13
5.1.1	<i>Norway</i> .....	13
5.1.2	<i>The Netherlands</i> .....	16
5.1.3	<i>Denmark</i> .....	17
5.2	COMPARISON OF THE PROPOSED CUT-OFFS. THE PROBLEM WITH STIMULANT DRUGS. ....	18
<b>6</b>	<b>RELATIONSHIP BETWEEN BLOOD CONCENTRATION AND EFFECT</b> .....	<b>20</b>
<b>7</b>	<b>WHICH SCIENTIFIC DATA CAN BE USED FOR THE ESTABLISHMENT OF “LOWER EFFECT LIMITS” AND “RISK THRESHOLDS”?</b> .....	<b>23</b>
7.1	WHICH SCIENTIFIC DATA CAN BE USED FOR THE ESTIMATION OF SUBSTANCE RELATED ACCIDENT RISK? .....	23
7.2	DISCUSSING PROS AND CONS OF DIFFERENT TYPES OF STUDIES.....	23
7.2.1	<i>Epidemiological studies</i> .....	23
7.2.2	<i>Experimental studies</i> .....	24
7.2.3	<i>Pharmacokinetic data (e.g. detection time)</i> .....	24
<b>8</b>	<b>ESTABLISHING A LIST OF CRITERIA FOR DEFINING CUT-OFFS</b> .....	<b>25</b>
8.1	SELECTION OF THE PSYCHOACTIVE SUBSTANCES FOR WHICH CUT-OFFS SHOULD BE DETERMINED.....	25
8.1.1	<i>Determination of substances, prevalence based</i> .....	25
8.1.2	<i>Should metabolites be included?</i> .....	25
8.1.3	<i>How to deal with combined consumption?</i> .....	26
8.1.4	<i>How to deal with the interval between the accident and the blood sampling?</i> .....	26
8.1.5	<i>How to deal with legal prescribed medicine use?</i> .....	26
8.1.6	<i>How to deal with tolerance?</i> .....	26
<b>9</b>	<b>REQUIREMENTS FOR ANALYTICAL METHODS</b> .....	<b>27</b>
9.1	ANALYTICAL METHODS USED IN DRUID .....	27
9.1.1	<i>Consensus on the substrate</i> .....	27
9.1.2	<i>Standardisation of collection and methods</i> .....	29
9.1.3	<i>Analytical methods</i> .....	30
9.1.4	<i>Quality assurance</i> .....	30
9.1.5	<i>Proficiency testing</i> .....	30
9.2	MEASUREMENT UNCERTAINTY .....	31
<b>PART 2: ANALYTICAL FINDINGS IN DRUID</b> .....		<b>35</b>
<b>10</b>	<b>TOXICOLOGICAL AND ANALYTICAL FINDINGS IN DRUID</b> .....	<b>35</b>
10.1	DETERMINING THE ORAL FLUID TO WHOLE BLOOD RATIOS FOR THE DRUID CORE SUBSTANCES .....	35
10.1.1	<i>Introduction</i> .....	35
10.1.2	<i>Sample collection and analysis</i> .....	35
10.1.3	<i>Data analysis</i> .....	35
10.1.4	<i>Results and discussion</i> .....	36

10.2	CONVERSION FACTORS FOR WHOLE BLOOD AND PLASMA .....	39
10.3	ESTIMATION OF EQUIVALENT CUT-OFF THRESHOLDS IN BLOOD AND ORAL FLUID.....	41
10.3.1	<i>Sample collection and analysis</i> .....	41
10.3.2	<i>Procedures for estimating equivalent cut-off concentrations</i> .....	42
10.3.3	<i>Results and Discussion</i> .....	42
10.4	THE BLOOD SPOT APPROACH .....	46
10.4.1	<i>Introduction</i> .....	46
10.4.2	<i>Materials and methods</i> .....	47
10.4.3	<i>Extraction and quantitative determination</i> .....	47
10.4.4	<i>Validation</i> .....	48
10.4.5	<i>Materials and instrumentation</i> .....	48
10.4.6	<i>Results and discussion</i> .....	49
<b>11</b>	<b>BIBLIOGRAPHY</b> .....	<b>55</b>

# 1 Executive Summary

## Part 1

This document makes recommendations for establishing cut-off levels for drugs in *per se* legislation for driving under the influence. It does not have the ambition to write a model *per se* drug-driving legislation, but based on the authors' experience, the experience in member states and Norway, the results of DRUID and scientific literature, it aims at giving pertinent considerations when a nation wants to determine *per se* cut-off levels.

Most European countries adhere to one of two possible approaches or definitions of the act of drug driving: 11 countries use the impairment approach, 8 countries use zero-tolerance or *per se* limits and 9 countries combine these two approaches into a two-tier system.

Until now, all countries that have *per se* legislation use analytical cut-offs, i.e. the lower concentrations that can reliably be determined by forensic laboratories. In some countries, these are the lowest limits of quantitation of the forensic laboratories, in other countries, they have been established by experts. In some countries, even if they are called analytical cut-offs, some consideration was given to a relationship with effects, e.g. by measuring only the active cannabis component THC, instead of the inactive metabolite, and using a cut-off that corresponds to a concentration after a single dose, when the drug still has effects.

There are three classes of substance thresholds: "Risk thresholds": concentrations in blood that indicate a certain accident risk or impaired driving. "Lower effect limits": the lowest concentration where an effect on driving is observed. And "Limit of detection": based on technical limitations in order to guarantee a valid and reliable analytical result and avoid false positive results.

In establishing thresholds, one must realise that the relationship between the concentration and the effect is not linear for most drugs, and that a given concentration could correspond to low effects (e.g. in a tolerant individual) or high effects (e.g. in a drug-naive subject).

The list of drugs to be included in *per se* legislation will depend on the situation in each country, e.g. the drugs that are most often found in the driving population or in drivers involved in an accident. Most countries have a very limited list of 10 substances or less. There is a consensus on not including medicinal drugs in the list. It is not reasonable to define cut-off values for patients in long-term treatment. Even high doses may lead to fewer effects because of tolerance.

Norway and the Netherlands recently tried to determine safe driving limits and they arrived to very similar values, e.g. 3 ng/mL THC or 48 and 50 ng/mL for MDMA in whole blood. Norway defined a risk threshold for THC, where the impairment is comparable to 0.05% BAC.

In determining "lower effect limits", stimulant drugs like amphetamines and cocaine pose a particular challenge. The correlation between drug concentration and risk of traffic accidents/impairment is variable or insufficiently documented. In experimental studies, at the (rather low) doses that were given, driving performance increases rather than decreases. However, in epidemiological studies indications of increased accident risk could be found. Since the available literature regarding the central stimulants did not provide evidence for dose-response effects, limits for graded sanctions were not suggested. Experimental and epidemiological results should be interpreted together for the determination of cut-offs.

Usually, inactive metabolites are not included in the legislation, except when the parent drug is unstable and is metabolised very rapidly.

Another issue to take into consideration is the rapid metabolism of drugs. Some drugs like THC have a very rapid metabolism, and if the delay between the stop or accident and the blood sampling is long, the concentration could have decreased markedly (based on a half-life of 1.4 hours, 3 ng/mL of THC decrease to 0.68 ng/mL after 3 hours). The "lower effect limits" should be established with this in mind. Another possibility, but less easy to implement, is that the lowest concentration that can be accurately measured (LOQ, limit of quantitation) is used instead of the lower effect limit when the sampling delay is longer than 2 or 3 hours.

The epidemiological studies in DRUID have shown that people very often use more than one drug. The question has been raised if the *per se* lower effect limits or the LOQ should be used when more than one drug (or alcohol) is detected. Some have recommended using the LOQs.

One of the risks of using lower effect or safe driving limits is that inevitably questions will be raised with respect to the dose that can be taken still remaining under the limit. One should realize that establishment of lower effect limits does not mean that one condones drug use. Moreover, in many countries (e.g. Sweden and Finland), people who are sanctioned for driving under the influence of narcotics will also be sanctioned for drug use, or sanctioned for drug use even if inactive metabolites are detected. But to achieve the compliance of the population a clear legislation should be implemented, which differentiates drug and traffic policy.

It is not a problem to limit the list of drugs in *per se* legislation to a few substances, if the *per se* law is combined with an impairment law, where all other impairing substances are covered. In this scenario, a quick and easy to enforce procedure exists for the most common drugs, and a more elaborate procedure exists for the less frequent cases, including medicinal drugs, combination of drugs, withdrawal, etc. It is not realistic to develop cut-offs for all the existing medicinal and recreational drugs. Moreover for new drugs, it might take some time before the different cut-offs have been established.

## **Part 2**

In DRUID many experimental results were gathered and a literature review was performed on conversion factors between plasma and whole blood and between whole blood (B) and oral fluid (OF).

The drug concentration ratios between oral fluid and blood (sometimes called oral fluid to blood conversion factors) were studied by collecting paired samples of oral fluid and whole blood from drivers in Belgium, Finland, Italy and Norway. Oral fluid to blood (OF/B) concentration ratios were calculated. Large variations were found between individuals; typically the coefficient of variation (relative standard deviation) was 50 to 100%. Therefore, conversion factors cannot be used to accurately estimate drug concentrations in blood based on drug concentrations in oral fluid. The estimated equivalent cut-off concentrations for oral fluid and blood were used for the calculations of drug prevalence (Deliverable 2.2.3) and for the odds ratio calculations (Deliverable 2.3.5)

Drug analysis in samples of oral fluid can be used to estimate the drug prevalence in blood if using equivalent cut-off concentrations. Three formulae were used for estimating equivalent cut-off concentrations using the average OF/B ratio, median OF/B ratio or percentile regression. To determine which formulae fit the original paired data best, the prevalence of samples above selected cut-off concentrations in blood was estimated using the formulae and compared with the actual prevalence in blood. The accuracies of the three procedures were calculated for the chosen cut-off concentrations in blood and for concentrations corresponding to 2.5 times and 5 times the analytical cut-off. The procedure with the least average percent deviation (in absolute value) from the actual number of subjects with drug concentrations above the cut-offs in blood was identified as the best one for each substance separately. Based on this 'equivalent' cut-offs were established for oral fluid.

The use of dried blood spots (DBS) has potential as a precise and inexpensive option for the determination of several analytes in small blood samples. The small sample volume of 100  $\mu\text{L}$  requires very sensitive techniques. By using LC-MS/MS, all analytes investigated could be determined with sufficient lower limits of quantitation (LLOQs). Evaluation data showed no significant differences in precision as well as lower limits of detection (LLODs) and LLOQs. Analysis of DBS is feasible with the advent of increasingly sensitive MS technologies such as LC-MS/MS. The DBS/B ratios were very close to 1.00, and the relative standard deviations  $\leq 8.56\%$ . The use of DBS in routine analysis will result in simplified handling during blood sampling, transport and storage as well as sample processing in the laboratory. DBS drug analysis can be regarded as a valuable and inexpensive alternative to determination from whole blood.

## Part 1: Per se limits

### 2 Introduction

As stated in the general conclusions of the Pompidou Group in June 2003 “The law enforcement and judicial authorities should have clear legislative and regulatory provisions, in line with which they can prosecute and convict individuals driving a vehicle whilst under the influence of psychoactive substances” (1)

In case of combating driving under influence of alcohol, legislative regulations and enforcement practices are clearly defined. Further, on *per se* limits for alcohol are based on scientific risk research that is a prerequisite to assure the compliance of the population with these regulations. Determining legislative regulations against drugged driving is more difficult as a variety of aspects has to be taken into account. First of all, one needs to determine which psychoactive substances should be prohibited from being consumed before driving. Of course it should be forbidden to drive under the influence of illegal drugs with impairing effects, but what should be recommended in case of taking prescription drugs? On the one hand, the impairing effects of some prescribed medicines are well known, but on the other hand the patient’s need for mobility should be respected. Furthermore, the disease itself may affect the driving behaviour even more and the use of medication could decrease this effect. Thus a balance between concerns about ensuring road safety and the therapeutic needs of an individual has to be guaranteed.

The main challenge in establishing legal regulations combating drugged driving is to define clear rules for detecting and sanctioning a drugged driver. Regarding alcohol a clear correlation between consumption, blood concentrations and the score of driving impairment is proved since several years, whereas up to now defining limits for combating drugged driving comprises a lot of challenges. To facilitate legal practice, laws against drugged driving are based on zero tolerance and consider drivers “impaired” if any amount of a listed drug or its metabolites can be detected in blood. But as the progressing technical improvement allows the detection of traces of substances long after they were taken and when the impairing effects have subsided, the need for setting lowest levels is indispensable. Therefore, use of the term “analytical threshold” for establishing substance thresholds for zero tolerance legislation should be revised and criteria should be established for cut-off determinations.

An important aspect is the relationship with drug use and drug possession laws. In many EU countries, use of narcotic drugs is illegal, and ‘one cannot have a legal level of an illicit drug’. Having cut-offs that are higher than the analytical cut-offs would give the impression that one is condoning narcotic drug use. This has been handled differently in member states: in Belgium, the law explicitly mentions that the observations made for enforcing the *per se* law cannot be used for prosecuting other drug-related offences, while in Sweden, someone sanctioned for driving under the influence of narcotic drugs, receives an additional sanction for drug consumption, and in Finland a finding of the cannabis metabolite THCCOOH in urine during the process of detecting drugged driving results in a sanction for drug use. But to increase the acceptability by the population, a clear legislation should be implemented, which differentiates drug and traffic policy.

In DRUID an expert group was established to work on these issues and in this document their recommendation towards determining cut-offs for *per se* legislation are presented. As analytical issues like determining which specimen to use (blood, plasma, saliva) for substance detection are important for defining cut-offs, the findings of the toxicological group in DRUID are represented in chapter 0.

### 3 Objectives

Besides addressing legal issues, cut-off values of psychoactive substances that can impair driving ability and hence present an increased risk of unsafe driving can only be established by focussing risk assessment based on empirical science as well as tackling analytical issues.

Therefore the following chapters will cover and discuss:

- Selection which scientific data are suitable for the estimation of substance related accident risk.
- Discussing pros and cons of different research methods
- Establishing a list of criteria for definition of a cut-off
- Selection of the psychoactive substances for which cut-offs should be determined
- Determination of substances that should be included, based on their prevalence
- How to deal with metabolites
- How to deal with combined consumption
- How to deal with legal prescribed medicine use
- Determination of analytical procedures
- Defining the analytical substrate
- Quality assurance of laboratory analysis
- Determining measurement errors
- Pros and cons of whole blood and plasma
- Saliva (oral fluid)
- Blood spots
- Conversion factors between the different body fluids

First of all the different legal approaches combating driving under influence and the definition in terms of wording for substance cut-offs are explained in the following.



## 4 Different legal approaches

### 4.1 Introduction

Most European countries adhere to one of two possible approaches or definitions of the act of drug driving: i.e. 1) the impairment approach (11 countries) or 2) zero-tolerance or *per se* limits (8 countries). Nine countries combine these two approaches and employ a two-tier system (2).

Impairment approach: if the driver shows clear symptoms of impairment whether in his personal behaviour or its driving style, he will be prosecuted. Most EU countries use fixed protocols to prove signs of impairment. An overview is given in D 3.2.2. One problem is that these so called drug recognition expert (DRE) programs are derived mostly from alcohol impairment detection protocols and are therefore not usable to detect all kind of drug effects.

Per se limits: if a drug is found in a driver's body fluid (blood, in some countries like Belgium, France, Spain and most of the Australian states, oral fluid) above a defined cut-off concentration he will be prosecuted. This approach facilitates prosecution. But for population compliance the cut-offs should be based on scientific risk analysis. This *per se* legislation is sometimes also called 'zero-tolerance legislation', but it is a confusing term. "Analytical thresholds" have not been defined according to technical issues but to drug concentrations of the active compound were an effect on driving ability might occur. They were often established taking the effects of the drugs into account to drug concentrations of the active compound were an effect on driving ability might occur. In most legislations the active ingredient of cannabis THC is the target drug, not the inactive THCCOOH, or setting a higher cut-off for benzoylecgonine (3).



Figure 1: Map of Europe showing the countries with *per se* (zero tolerance), impairment legislation and two-tier system. (2)

Two-tier system: *Per se* limits (for drugs limits for zero tolerance) are combined with an impairment approach. This system allows combining the advantages of the two legal regulations. As research has up to now not proposed a clear-cut solution to link substance consumption to distinct levels of impairment this two-tier system seems to be the most favourable one to combat driving under psychoactive influence other than alcohol. Moreover, this system allows combining a less severe sanction when

drugs are present above the *per se* limit and a more severe sanction when the driver was impaired. This is e.g. the case in Germany and in the proposed Norwegian *per se* legislation. The situation in the different EU countries is illustrated in Figure 1.

## 4.2 Definition of cut-offs

One may differentiate four classes of substance thresholds:

“Risk thresholds”: Psychoactive substance concentrations in blood are indicating a certain accident risk respectively impaired driving. In DRUID Task 1.3 risk thresholds for psychoactive substances are determined showing the same accident risk as a blood alcohol concentration (BAC) of 0.5 g/L.

“Lower effect limit”: The cut-off has been set so that the concentration is to the lowest concentration where an effect on driving is observed. Substance detection below this threshold does not imply recent psychoactive substance consumption or being under the influence. This limit is comparable to a BAC of 0.2 g/L.

“(Lower) Limit of quantification (LLOQ or LOQ)”: the smallest measured content from which it is possible to quantify the analyte with an acceptable level of accuracy and precision. This threshold is based on technical limitations and should guarantee a valid and reliable analytical result, also avoiding false positive results.

“(Lower) Limit of detection (LLOD or LOD)”: is the smallest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. An alternative definition is that LOD is the lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise or LOD is the smallest amount or concentration that can be readily distinguished from zero and be positively identified according to predetermined criteria and/or levels of confidence (4).

New analytical technologies are more and more sensitive and enable detecting very small quantities of substances. An increasing/inherent problem will be then that a positive blood sample will not always imply traffic safety risks i.e.: when an illicit or medicinal drug was consumed many hours or days before and psychoactive effects are no longer present. A second problem is that with continuously decreasing analytical thresholds, legal thresholds cannot be established. To overcome this problem, “lower effect limits” were proposed. This should define the cut-off value for zero-tolerance legislation, based on scientific knowledge about the non-impairing effect of low concentrations. Cut-offs must be defined below which impairment can be excluded and not for technical limits. However, low limits make sense, e.g. for stimulant drugs. While the DRUID on-road driving studies did not show an impairing effect at therapeutic doses of stimulants; the risk increases if they are taken in combination with sleep loss or alcohol. Deliverables 1.1.2.b and D1.2.1 concluded that impairment may be detected only at very high doses, but also that the effects after an acute intoxication with amphetamines are frequently characterised by sleepiness and exhaustion, which are, of course, of special relevance for traffic safety. Some studies have shown that most accidents occur when the effect wears off and fatigue sets in. Logan concluded that “methamphetamine at any concentration is likely to produce symptoms that are inconsistent with safe driving (5). Although the Task 1.4 expert group (of which the first author of this deliverable was not a member) decided not to define cut-offs for post acute effects because one may not sanction fatigue based on drug or alcohol consumption, this doesn’t change the fact the most countries have used low cut-offs in their legislation.

When the *per se* legislation was introduced in Germany, the following considerations were made (3): *“However, for the definition of the limit values these concentration ranges alone are not crucial, but security of the proof with consideration of disturbance is determining. If necessary the limit value should encompass a concentration range of the substance, with which the endangerment of traffic safety is usually enclosed. The proof must include the fact that the subject has consumed an impairing drug and that the time of consumption is sufficiently close. One must be able to quantitate the drugs at the limit concentrations with coefficients of variation that are representative and that have been determined by interlaboratory comparison. Values that can be determined quantitatively sufficiently exactly must be two standard deviations above the limit of determination. Sometimes however values can be*

sufficient, which lie over the safe detection limit, because for individual substances individual aspects must be considered. (...)

*Because of its instability in vitro, cocaine can only rarely be detected. Even the use of preservatives like sodium fluoride can only partially stabilise the analyte in the blood between sampling and freezing in the laboratory. As a consequence, the most abundant metabolite benzoylecgonine must be determined. Since it is inactive and excreted more slowly, the limit value that can be specified will be set substantially higher than it would be based on the possibility to analytically determine the parent drug. Also in the case of heroin a determination of the originally consumed material via the parent drug or 6-acetylmorphine is not possible so easily. However morphine, an active metabolite is available.*

*To confirm the correctness of the findings, additional criteria can be introduced, that confirm the plausibility of the result of the analysis. This can occur by means of the determination in a legally defensible way of specific metabolites and/or the exclusion of certain influence factors. Such plausibility criteria do not have an influence on the height of the limit value.*

*With all the described factors the limit values meet the quality requirements for a legally defensible proof. As analytical limit values they are not to be equated with an effect limit for the analytes, although they cover "drug effect" prospectively. Which should be understood as "drug effect" requires if necessary more detailed discussion. If only whole blood is available for the determination (no serum) is present, other limit values or conversion factors must be specified (3)"*

## 5 Overview of European regulations concerning *per se* legislation

A DRUID questionnaire concerning legal regulations regarding drugged driving and legally defined cut-offs for illegal psychoactive substances was distributed in the European member states, Norway, Switzerland and Croatia. The reply was complemented with the official data of the EMCDDA and data available to the authors (see Table 1).

Table 1: Legal regulations applied in each country

Countries	Legal approach related to driving	
	Impairment	Zero tolerance
Austria	X	
Belgium	X	X
Bulgaria		X
Croatia		X
Cyprus	X	Coming soon
Czech Republic	X	X
Denmark	X	X
Estonia		X
Finland	X	X
France		X
Germany	X	X
Greece	X	
Hungary		X
Ireland	X	
Italy		X
Latvia		X
Lithuania		X
Luxembourg	X	
Norway	X	Coming soon
Poland		X
Portugal		X
Slovakia	X	X
Slovenia		X
Spain	X	
Sweden		X
Switzerland		X
The Netherlands	X	Coming soon
United Kingdom	X	

Four of the 17 countries defined cut-offs for illegal drugs in plasma (resp. serum), whereas all others defined their cut-offs in whole blood. This has to be taken into account when comparing the data of table 2. In some countries (e.g. France and Belgium), the cut-offs are mentioned in the law, while in

other countries, it is based on the recommendations of an advisory group (Germany), or the lower limit of quantitation of a national forensic laboratory (Sweden, Finland).

Table 2: Cut-offs per substance applied in each country. Some cut-offs are defined in the law, some others are the ones used by the forensic laboratories

Countries	THC	THC COOH	Ampheta- mine	Metham- pheta- mine	MDMA	MDA	MDEA	Cocaine	Benzoyl- ecgonine	Morphine
Belgium	1		25		25			25	25	10
Denmark	1		20	20	20	20	20	20		10
Finland*	1	5	25	25	25	25	25	10	10	2.5
France	1		50	50	50	50	50	50		20
Germany	1		25	25	25	25	25	10	75	10
Great Britain	2	10	25	50	50	50	50	50	50	25
Greece	1		25	25	25	25	25	10	10	10
Hungary	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0	> 0
Ireland	2	5	10	10	10	10	10	2	10	10
Italy***	0	0	0	0	0	0	0	0	0	0
Italy**	2	2	20	20	20			2		10
Italy LOQ	0.5	0.5	20	20	20			10	10	5
Luxembourg	2		50	50	50	50	50	50	50	20
Poland LOQ	2	50	50	50	50	50	50	50	50	20
Portugal	3	5	5	5	5	5	5	5	5	5
Slovenia	0.3	5	20	50	20	50	50	10	5	50
Sweden	LLQ	LLQ	LLQ	LLQ	LLQ	LLQ	LLQ	LLQ	LLQ	LLQ
Switzerland	1.5 + 30%		15 + 30%	15 + 30%	15 + 30%		15 + 30%	15 + 30%		15 + 30%

Impairment
Zero tolerance
Both

Plasma/serum
Whole blood

\* cut-offs used by the National Institute of Public Health (National Institute for Health and Welfare) until the 2009

\*\* scientific guidelines by Forensic Toxicologists

\*\*\* by legislation

## 5.1 Discussing different regulations in Norway, The Netherlands and Denmark

Recently, three EU countries determined cut-offs for *per se* legislation. In this part, we review the procedures and criteria used, before we compare the cut-offs that were obtained.

### 5.1.1 Norway

Since 1936, Norway has an impairment-based law for driving under the influence (DUI) of alcohol (ethanol). The impairment limit for alcohol is now 0.2 g/L and graded sanctions are given for higher blood alcohol concentrations, with limits corresponding to levels of 0.5 g/L and 1.2 g/L. For non-alcohol drugs Norway has an impairment-based law regarding DUI. In these cases, the judicial process requires an expert witness statement to evaluate driver impairment and to compare the degree of impairment to corresponding BACs.

### 5.1.1.1 A harmonisation of the system for DUI of alcohol and non-alcohol drugs in Norway

The Norwegian Ministry of Transport and Communications sought to establish a new limit based penal process in DUI cases involving drugs not subject to a prescription from a physician. The new system should be based on a zero tolerance policy against impaired driving, disregarding individual tolerance and drug-disease interactions. The Ministry asked an advisory group to suggest a system for handling DUI of the most frequently used non-alcohol drugs that lead to increased risk of traffic accidents and legislative concentration limits for impairment of non-alcohol drugs corresponding to a BAC of 0.2 g/L (“impairment limits”) and BACs of 0.5 and 1.2 g/L (“limits for graded sanctions”).

### 5.1.1.2 Impairment limits

This limit corresponds to the degree of impairment comparable to a BAC of 0.2 g/L. The proposed limits for the drugs are given in Table 3.

This limit should represent a threshold where higher concentrations in blood are likely to induce impairment comparable to those accompanying blood alcohol concentrations of 0.2 g/L.

For alcohol a concentration of 1 g/L is assumed to lead to euphoria and impairment, and the legal limit for alcohol in Norway is 0.2 g/L. The low limit for alcohol is thus 1/5 of a concentration of alcohol seen after a typically euphoric dose. Due to lack of scientific documentation in this low concentration area, the group estimated 1/5 of the concentration of a “drug-dose” to find the low limits. Corrections were made due to factors regarding therapeutic drug concentrations, half-lives etc.

### 5.1.1.3 Limits for graded sanctions

These limits (Table 3) correspond to the degree of driving impairment related to BAC levels of 0.5 g/L and 1.2 g/L respectively.

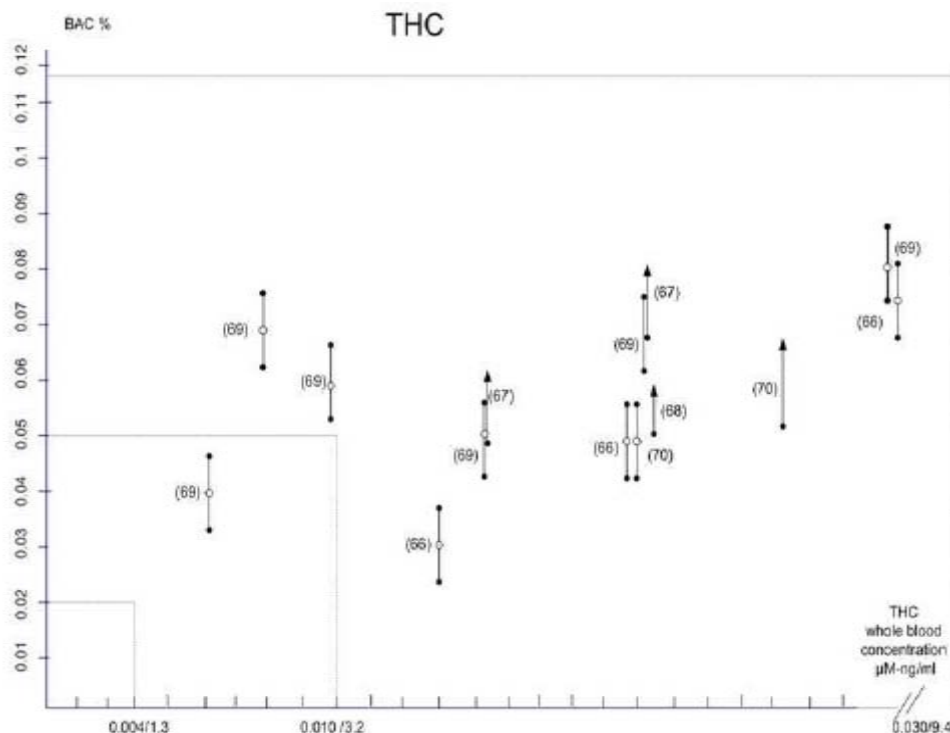


Figure 2: Example of a diagram showing the correspondence between the effects of THC and ethanol. A cut-off of 0.2 g/L ethanol corresponds to 1.3 ng/mL THC in whole blood (from Vindenes et al., manuscript in accepted for publication in Forensic Science International.)

Impairment limits corresponding to the 0.5 and 1.2 g/L limit for alcohol were defined for the drugs where scientific evidence showed a dose-response relationship for impairment. To suggest specific limits for each drug, the literature has been searched to find the “best/most relevant” publications re-

garding traffic relevant impairment, according to certain criteria. Impairment on the tests at different drug concentrations was compared to alcohol impairment, and plotted in diagrams for each substance. From these diagrams the limits corresponding to impairment at 0.5 and 1.2 g/L alcohol were suggested.

A total of 45 studies were included. The results for each of the included drugs were plotted in separate diagrams. Figure 2 shows an example of such a diagram for THC. Impairment at the drug concentrations tested was compared to BAC levels tested in the same study. Since a low number of doses of alcohol have been investigated, arrows indicate if the impairment on each test was more or less pronounced than at the tested BAC. An estimated effect interval was drawn if impairment was estimated to be in the same range for the investigated drug concentration as for alcohol. The open circles represent the actual concentrations of the tested substances. Some of the studies conducted tests at different time points after drug ingestion, and test results at different concentration levels were plotted in these instances. Some studies included various relevant psychomotor tests, and summaries of the impairment from these tests were plotted for each study.

#### 5.1.1.4 Discussion

*Per se* legislation limits have been proposed for a selection of frequently detected psychoactive drugs in DUI cases in Norway. Due to a lack of scientific evidence, a pragmatic approach has been used. The scientific literature for comparing risk of traffic accidents and reduced performance at different concentrations levels after ingestion of drugs and alcohol is sparse, and the suggested limits may be subjected to debate. Since the aim of this work was to harmonise the judicial process involving alcohol and non-alcoholic drugs, the working group found this method acceptable for the suggested legislative limits.

Limits for graded sanctions were not suggested for the central stimulants, due to lack of scientific evidence for the relationship between a drug concentration and risk of traffic accident/impairment. From the relevant literature regarding central stimulant agents, several of their effects may not be compatible with safe driving, and even low concentrations might lead to substantial reduction in driving ability.

Benzoylcegonine was frequently detected in DUI cases. This cocaine metabolite is inactive, and was therefore not considered a candidate for legislative limits

More details on the new Norwegian legal limits and impairment limits will be published (Vindenes et al., manuscript in accepted for publication in Forensic Science International.).

Table 3: proposed limits for DUI in Norway.

DRUGS	Low limits (ng/mL in whole blood)	Impairment limits comparable to 0.5 g/L BAC (ng/mL in whole blood)	Impairment limits comparable to 1.2 g/L BAC (ng/mL in whole blood)
Benzodiazepines and benzodiazepine-like			
Alprazolam	3	6	15
Clonazepam	1.3	3	8
Diazepam	57	143	342
Fenazepam	1.8	5	10
Flunitrazepam	1.6	3	8
Nitrazepam	17	42	98
Oxazepam	172	430	860
Zolpidem	31	77	184
Zopiclone	12	23	58
Cannabis			
THC	1.3	3	9

DRUGS	Low limits (ng/mL in whole blood)	Impairment limits comparable to 0.5 g/L BAC (ng/mL in whole blood)	Impairment limits comparable to 1.2 g/L BAC (ng/mL in whole blood)
Central stimulants			
Amphetamine	41	*	*
Cocaine	24	*	*
MDMA	48	*	*
Methamphetamine	45	*	*
GHB			
GHB	10 300	30 900	123 600
Hallucinogens			
Ketamine	55	137	329
LSD	1	*	*
Opioids			
Buprenorphine	0.9	*	*
Methadone	25	*	*
Morphine	9	24	61

\*Limits have not been suggested because the correlation between drug concentration and risk of traffic accidents/impairment is variable or insufficiently documented. For instance, marked impairment can be seen at low concentrations for substances like amphetamine and methamphetamine, in particular some time after substantial drug intake.

### 5.1.2 The Netherlands

Up to now in the Netherlands only legal limits for alcohol (novice drivers: 0.2 g/L, others: 0.5 g/L BAC) are determined. But as in Norway an expert group working on the definition of legal limits for particular drugs was implemented and is proposing new guidelines. It was decided to define limits only for single drug use, related to effects in new users and the time period between stop by the police and blood sampling should be limited. The cut-offs are understood as an impairment approach, as they are related to effects. In case these cut-off values are reached, no "additional" sign of impairment is required.

For 10 substances cut-offs for blood and plasma have been defined by using different sources of information (e.g. published research results, (6, 7)), see Table 4.

Table 4: Cut-offs in plasma and whole blood proposed by the Dutch working group.

Drug	Analyte that is measured	Cut-off in plasma □ (ng/mL)	Cut-off in blood □ (ng/mL)
Amphetamine	Amphetamine	50*	50*
Methamphetamine	Methamphetamine	50*	50*
MDMA	MDMA	50*	50*
MDEA	MDEA	50*	50*
MDA	MDA	50*	50*
Cannabis	THC	5	3
Cocaine	Cocaine	50	50
Heroin	Morphine	20	20



Morphine	Morphine	20	20
GHB, GBL, 1,4-butanediol	GHB	10 mg/L	10 mg/L

For defining the cut-offs of stimulants a pragmatic approach was chosen. As stimulant use is often combined with sleep deprivation, which causes driving impairment the cut-offs are fixed following the pharmacokinetic profile of regular stimulant use after a night of sleep loss. Because all stimulants are acting in the same way, and there are no prominent differences in their potency, the same cut-offs were chosen. In addition the setting of use plays an important role. The cut-off for THC was derived from publications as the comprehensive review of Grotenhermen (8).

One criticism stated from some DRUID partners was that the long half-life of stimulants has to be considered thus it can be assumed that at these low cut-offs no impairment can be observed anymore. However, the rationale given in Norway and The Netherlands for the choice of the cut-offs addresses this criticism. The other point is that the main reason for driving impairment is the sleep deprivation and not the drug intake itself. But the question can be raised if this lack of sleep could be possible without the use of drugs.

One should realize that despite the fact that these cut-offs have been determined based on the most recent literature data, they are still approximations, and a having a concentration of drug under the cut-off does not automatically mean that the drug cannot be the explanation for the impairment observed in a subject. But this is also the case for alcohol, where some subjects can be impaired even when their blood alcohol concentration is lower than 0.5 g/L, which is the legal limit in most EU member states.

### 5.1.3 Denmark

In Denmark all illegal and legal drugs with abuse potential (e.g. opioids) are forbidden in the traffic above a fixed concentration limit in whole blood. Legal drugs are excepted in case one has a prescription and one is judged able to drive in a sure manner (investigated by a medical doctor).

For therapeutic drugs, e.g. morphine, the limits were selected as the lower therapeutic limits taken from the literature (in practice serum concentrations; most drugs have about equal distributions in blood/serum), e.g. for morphine, e.g. 0.010 mg/kg. As sources, the compilation by Schulz & Schmoldt (9), and other sources in textbooks were used. Schulz & Schmoldt is a source that is fully referenced and so this source was used in most cases.

Concerning illegal drugs with no therapeutic use, the lower limit for pharmacological effect was used if available, or a concentration level documented to correspond with intake of usual abuse doses (e.g. drugs as cathine or cathinone). For the most frequently abused drugs that have limits established in other countries, these limits were also taken into account, e.g. for THC, although it is known that the limit for actual proven disability to drive safely is a little higher than 0.001 mg/kg in case of THC. However, for THC one should also be aware of the fast elimination during the first few hours following drug use that often will give a low concentration at the time of sampling. Thus, given a concentration of 0.001 mg/kg it is likely that a level associated with impaired driving was present in the preceding hours (there may be some special considerations in relation to chronic abuse).

Metabolites (inactive) are not included because the Danish legislation is confined to active substances. This is a limitation for e.g. cocaine, where benzoylecgonine cannot be used.

In the lab, 50% is added to the legal limits to take measurement uncertainty into account and deliver an answer: whether a drug is present or not present in relation to these limits.”(Prof. Kristian Linnet, Department of forensic medicine in Copenhagen, personal communication).

## 5.2 Comparison of the proposed cut-offs. The problem with stimulant drugs.

Table 5 compares the proposed cut-offs equivalent to 0.5 g/L ethanol. One can observe quite a good agreement between the cut-offs proposed in the Netherlands and Norway, except for cocaine (double in the Netherlands compared to Norway) and GHB (3 times higher in Norway). In the document of Prof. Berghaus et al (D1.1.2b), the value for THC is lower than that obtained in Norway and The Netherlands. This limit is a raw value derived from experimental studies with the corresponding impairment at 0.5 g/L ethanol. For the definition of a legal limit one has to add a measurement error and thus one reaches the same values as Norway and the Netherlands.

Regarding the effect of stimulants it is written in D1.1.2b (10) that “at least with respect to driving-related performance there seem to be no statistically significant impaired effects that exceed the 15% threshold” (limit equal to impairment seen with 0.3 g/L ethanol). They also state “Hence, concerning driver fitness as tested with “normal” doses (40 mg - 125 mg) in experimental studies, the risk potential of ecstasy comprised during the time of action primarily not the impairment of performance.

Summary of DRUID findings regarding stimulant effects:

- Experimental studies: MDMA (25, 50 and 100 mg) and dexamphetamine (10, 40 mg) did not impair performance in a standardized on-road test of driving performance (SDLP; a measure of lane-keeping performance). But the stimulating effects of MDMA and amphetamines are not sufficient to overcome or compensate driving impairments produced by concomitant alcohol use or sleep deprivation (D 1.2.1). A meta-analysis of experimental studies revealed that there are more findings of performance improvement than of performance impairment (D 1.1.2b) under influence of stimulating drugs. But consumers of stimulating drugs may not be aware of post acute fatigue and thus need to be educated about this effect and its possible implications on driving safety (D 1.2.1).
- Stimulant use is often accompanied by sleep deprivation. Sleep deprivation itself causes the same degree of impairing effects as the influence of 0.8 g/L BAC (D 1.2.1).

Epidemiological studies:

- The odds ratios of responsibility for amphetamines, cocaine and opiates are not significantly different from 1, which indicates that the probability of being responsible for a fatal crash is not increased (D 2.3.2).
- The odds ratios of the case control studies indicate a significantly increased accident risk with high concentrations of amphetamines. This result should be interpreted with care, because the numbers of cases and controls are low and by adding or subtracting one case the OR became not significant (D2.3.5).

On the other hand non-experimental studies and case reports performed before DRUID revealed negative effects of amphetamines in terms of driving safety in the effective phase such as euphoria, agitation and confusion, increased risky behaviour, overestimation of one's own possibilities, restricted critical thinking and inner restlessness. Furthermore, the effects after an acute intoxication with amphetamines are frequently characterised by sleepiness and exhaustion, which are, of course, of special relevance for traffic safety. These circumstances seemed to comprise at least a certain risk for a safe participation in motor traffic. But on the one hand there was no information about the frequency of such effects and on the other hand one has to ask why these deficits did not lead to severe impairment in performance tested in experimental studies.

It seemed that the experimental research as done currently is at the frontier of its possibilities in this situation. May be it would be of interest to compare in an experimental approach the point of time and the concentration of amphetamine in blood when the increased performance not further overlaps the increased risky behaviour or the overestimation of one's own capacity (quote from Deliverable 1.1.2b). In the Norwegian evaluation, a similar conclusion (since the available literature regarding the central stimulants did not provide evidence for dose-response effects, limits for graded sanctions were not suggested) was made.

Table 5: Comparison of the proposed cut-offs in whole blood in Norway (impairment limit corresponding to 0.5 g/L ethanol) and the Netherlands and Denmark with the cut-offs suggested by Prof. Berghaus for some selected drugs. All concentrations are given in ng/mL (or µg/L).

	Norway	The Netherlands	Denmark	Berghaus (10) DRUID D1.1.2b
Amphetamine	41*	50	20	NR
Methamphetamine	45*	50	20	NR
MDMA	48*	50	20	NR
THC	3	3	1	2**
Cocaine	24*	50	20	NR
Morphine	24	20	10	

\* Limits are equivalent to 0.2 g/L ethanol. Limits equivalent to 0.5 g/L ethanol have not been suggested because the correlation between drug concentration and risk of traffic accidents/impairment is variable.

\*\* Mentioned as 3.8 ng/mL in serum after smoking, 3.7 ng/mL in serum after oral consumption

NR: not reached: no studies showed that impairment was an effect of acute amphetamine or cocaine use. See text for more details.

## 6 Relationship between blood concentration and effect

Many lay people suppose that there is a good relationship between drug concentrations in blood and the effect (Figure 3). However this is rarely the case. It is more or less the case for alcohol, but even there, there are some differences. Figure 4 shows that in a very old publication, the maximum intoxication score for alcohol comes earlier than the peak concentration and that the intoxication decreases more rapidly than the blood alcohol concentration. This is also illustrated in the concentration-effect relation shown in Figure 5.

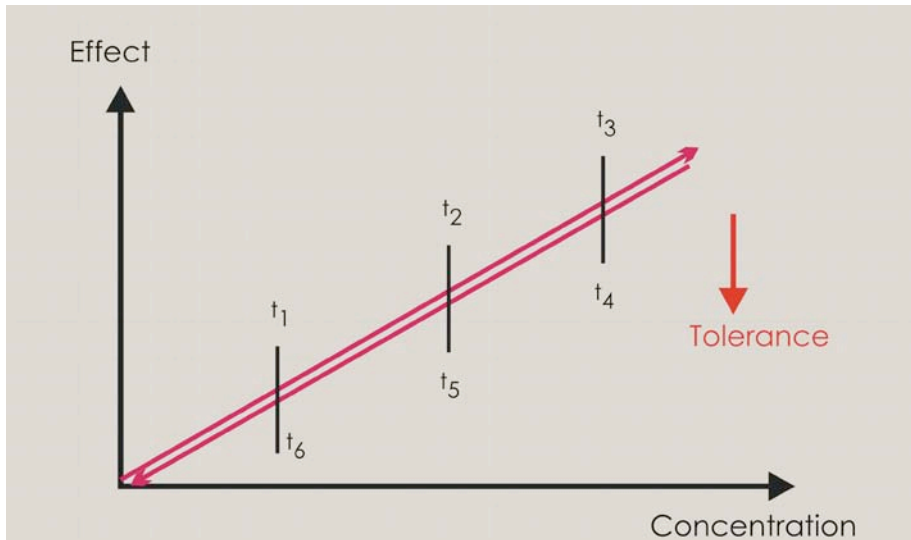


Figure 3: Ideal concentration-effect curve.

In principle, the effect should be related to the concentration in the brain or at the site where the drug exerts its effects.

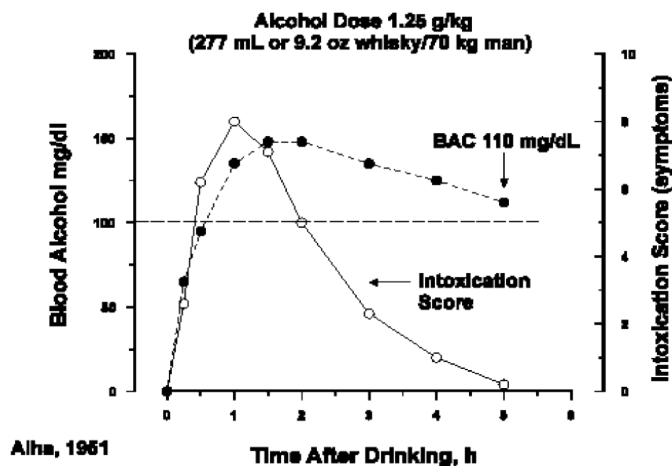


Figure 4: Comparison of the blood alcohol concentration and the symptoms of intoxication. (11)

The effect of a drug, e.g. the 'high' experienced when using a drug, is more related to the rate of increase in the concentrations than to the absolute concentration itself. The abuse liability of a drug is enhanced by rapidity of onset because effects that occur soon after administration are more likely to initiate the chain of events that leads to loss of control over drug-taking. The pharmacokinetic variables influence the time it takes a drug to reach critical receptor sites in the brain. The history of cocaine use illustrates the changes in abuse liability of the same compound, depending on the form and the route of administration. When coca leaves are chewed, cocaine is absorbed slowly through the

buccal mucosa. This method produces low cocaine blood levels and correspondingly low levels in the brain. The mild stimulant effects produced by the chewing of coca leaves have a gradual onset, and this practice has produced few, if any, behaviour problems despite use over thousands of years by natives of the Andes mountains. Beginning in the late 19th century, scientists isolated cocaine hydrochloride from coca leaves and refined the technology for extraction of pure cocaine. Cocaine could be taken in higher doses by oral ingestion (gastro-intestinal absorption) or by absorption through the nasal mucosa, producing higher cocaine levels in the blood and a more rapid onset of stimulation. Subsequently, it was found that a solution of cocaine hydrochloride could be administered intravenously, giving a more rapid onset of stimulatory effects. Each newly available cocaine preparation that provided greater speed of onset and an increment in blood level was paralleled by a greater likelihood of addiction. In the 1980s, the availability of cocaine to the American public was increased further with the invention of crack cocaine. Crack, sold illegally and at a low price (\$1-3 per dose), is alkaloidal cocaine (free base), which can be readily vaporised by heating. Simply inhaling the vapours produces blood levels comparable to those resulting from intravenous cocaine owing to the large surface area for absorption into the pulmonary circulation following inhalation. The cocaine-containing blood then enters the left side of the heart and reaches the cerebral circulation without dilution by the systemic circulation. Thus, inhalation of crack cocaine is much more addictive than chewing, drinking, or sniffing cocaine. Inhalation, with rapid attainment of effective drug levels in the brain, also is the preferred route for users of nicotine and cannabis. (12)

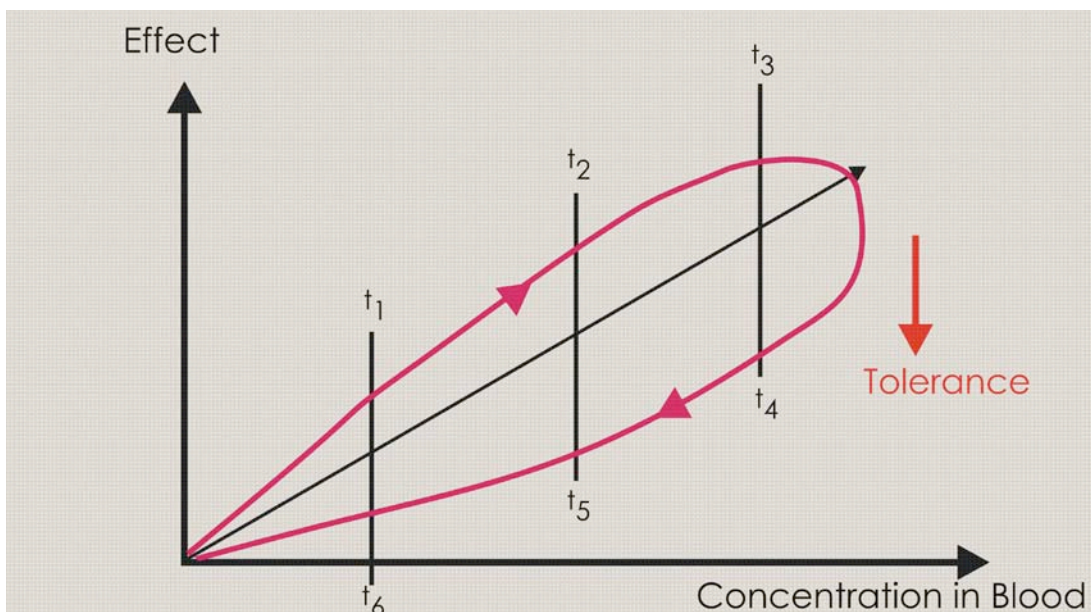


Figure 5: Concentration-effect relationship for alcohol. Immediately after intake, the effect increases more rapidly than the blood concentration, while the reverse is true later on ( $t_4 - t_6$ ).

For some drugs like cannabis, the effects come later than the peak blood concentration, a phenomenon that is called hysteresis (Figure 6). From this figure, it appears clearly that one cannot expect a good relationship between the concentration of the drug in the blood and the effect. A low concentration in the blood can correspond to a severe effect or to a relatively minor effect. This being said, the phase corresponding to  $t_1$  to  $t_3$  in the figure is very short, corresponding to the actual smoking of the joint. The period of most relevance for driving is  $t_4$  to  $t_6$ , which shows that the effect continues for a long time even if the concentration decreases, hence the placing of the lower effect limit at the descending part of the curve.

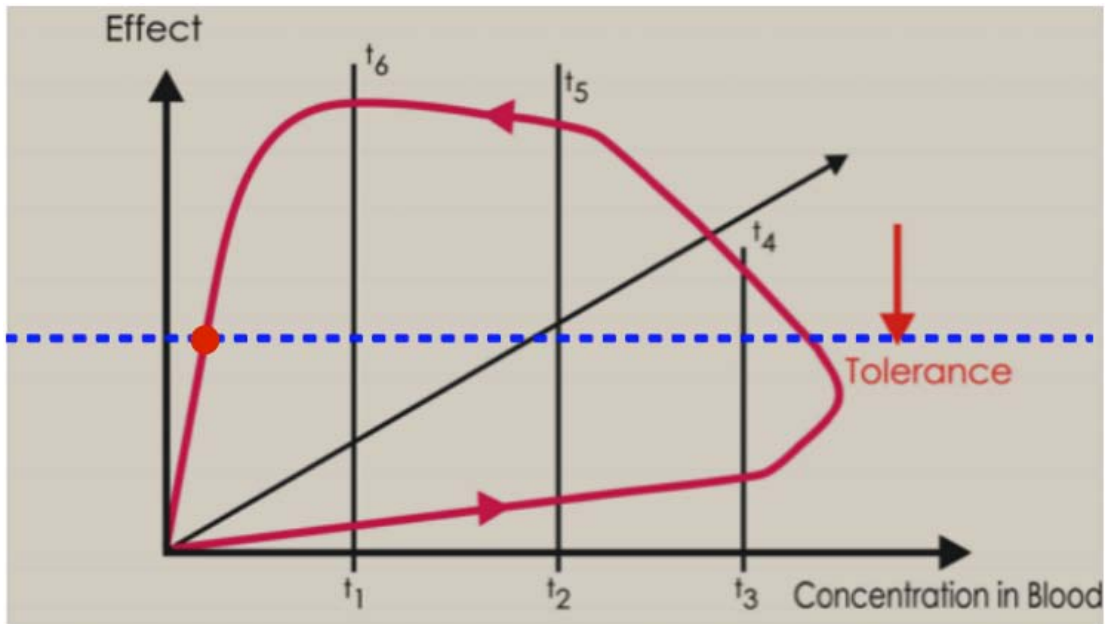


Figure 6: Example of a hysteresis curve for cannabis, where after intake ( $t_1$  to  $t_3$ ) the blood concentration increases more rapidly than the effects. Later on, ( $t_3$  -  $t_4$ ) the concentration is stable but the effect increases. The concentration at time  $t_1$  and  $t_6$  corresponds to low effects ( $t_1$ ) or maximal effects ( $t_6$ ). This illustrates the problem of setting a cut-off: for a similar affect (blue dashed line), the concentration corresponding to the effect could be low or high. As  $t_1$  -  $t_3$  is a short period corresponding to smoking, the lower effect cut-off should be placed at the left-most crossing point of the red curve and the dashed blue line (red circle).

## **7 Which scientific data can be used for the establishment of “lower effect limits” and “risk thresholds”?**

### **7.1 Which scientific data can be used for the estimation of substance related accident risk?**

Setting a concentration threshold for a psychoactive substance in traffic means to presume that a driver exceeding this limit is likely to be unfit to drive and may be prosecuted without further evidence. With respect to this consequence, it becomes immediately evident that on the one hand the decision for a concentration limit must be based on empirical results about the impairing potential but on the other hand cannot be restricted to those empirical results alone. Especially in case of psychoactive substances, the behavioural variance between persons is extremely high. Therefore, the relation between substance concentration and behavioural impairment (consequently driving fitness) will only be a probabilistic one. The higher a concentration limit is, the more likely impairment can be assumed for all drivers. As a consequence, the result of empirical studies – either epidemiological or experimental ones – is only one part of the discussion about reasonable concentration limits, albeit an important one.

To justify a limit, other questions must be answered like: How precise can concentrations be measured? How large is the deterrence effect of *per se* limits? Is the accepted risk for therapeutic drug-induced impairment the same as for recreational drugs? Therefore, the following compilation and discussion of empirical results about substance-induced impairment will only yield a framework for the much broader discussion about setting concentration limits.

### **7.2 Discussing pros and cons of different types of studies**

#### **7.2.1 Epidemiological studies**

The most relevant information, besides political or ethical considerations, in order to determine thresholds is the information about the accident risk in traffic dependent on different concentrations of single substances. Direct information about the accident risk in traffic can only be gained by conducting epidemiological studies. Representative studies on prevalence in accident-free and accident populations are difficult and expensive. Especially for substances with a low exposure rate in the population, a huge sample ought to be examined in order to get reliable estimations. Thus, for most of the substances, either legal or illegal, the data necessary for calculating risk indices are missing or incomplete, which leads to substantial problems for the estimation of traffic risks. Therefore experimental data should fill the knowledge gaps.

It is important to know the limitations of the single epidemiological study type in order to be able to weigh the results regarding their validity. Relative merits of experimental and epidemiological approaches are already described in literature (13, 14), thus in the following some remarks will be made.

Case control-studies are conducted mostly in the form of roadside prevalence data compared with accident data derived from hospital cases. Here it is important that the substance concentrations in the same body fluids are measured. There are very few data on the relationship between the presence and concentration of drugs and alcohol and the severity of the accident. Smink et al. (15, 16) found that there is no clear association between use of psychoactive substances and the severity of crash-related injury. Also all toxicological limitations have to be taken into account when interpreting the risk estimations of an epidemiological study e.g. how much time after the accident or even death was the sample drawn?

By comparison of odds ratios from these case control studies with those of responsibility studies one has to suggest if the risk estimation of the same substance concentration will lead in both study types to the same odds ratio or if methodological differences may influence the OR. To get at least a feeling of comparability and integrability of study results resulting from different methodologies, a reference curve is helpful. For instance alcohol data delivered with these different study methodologies could be used as the gold standard (this will be the case in DRUID D1.3.1).

In cases where low prevalence epidemiological data do not allow risk calculation (odds ratios) of different concentration ranges from single psychoactive substances the results of experimental studies should be taken into account.

### **7.2.2 Experimental studies**

Experimental studies allow substance concentration dependent investigations on driving behaviour. One of the most criticisms is that it seems doubtful to draw conclusion on driving impairment from some driving parameters as for instance the SDLP (standard deviation of lateral position). Reference values from alcohol data may help to interpret the drug effects on driving parameters, but one has to keep in mind that alcohol and drugs do not act in the same way especially if drugs belong to the group of stimulants. In several experimental studies stimulants do not show impairing effects that are related with safe driving behaviour, but can we follow only on basis on these studies that under stimulant influence driving is safe? One limitation in experiments is that very low doses are used for ethical reasons and only higher drug concentrations will deliver impairing effects.

Concerning the effects of stimulants the DRUID expert group agreed on:

“Post acute effects”, that means impairing effects not caused by the drug action but caused after the drug action by the symptoms of “hang over” will be excluded for the determination of cut-offs.

This is similar with the procedure of determining the alcohol limit. If impairment would be measured the next morning after a lot of alcohol consumption, impaired performance could be detected below a BAC of 0.5 g/L. For alcohol we only sanction its presence in blood or breath. This could be the same for all illegal drugs, despite the fact that many substances afterwards cause deterioration as well (post-acute phase).

Concerning experimental data on medicinal drug effects one has to see that medicinal drugs have mainly been tested in drug naive, healthy volunteers, whereas studies investigating the effect of illegal drugs are conducted with occasional drug users.

The DRUID expert group decided that for illegal drugs as well as for illegal medicine use all studies investigating the effect of chronic consumption should be neglected. Cut offs should be defined for occasional users not for regular ones as they develop habituation on substance levels which are too high for their appliance on occasional users.

Regarding the application of legal regulations one has to differentiate the prescribed and intended intake of medicines from illegal or not intended use of medicines.

### **7.2.3 Pharmacokinetic data (e.g. detection time)**

In case not enough scientific studies – whether epidemiological nor experimental - are available, pharmacokinetic data could support the determination of cut-offs. For defining the pharmacokinetic profile of medicines one should take the usual prescribed dose and for defining the profile for illegal drugs one should figure out the usual consumption amount of occasional drug users. The cut-off could then be set at a certain time after use, e.g. the duration of the effects or pragmatically, four times the half-life of the substance. This approach was chosen by the countries described in 5.1. Denmark, Norway and the Netherlands defined stimulant/cocaine cut offs on the basis of pharmacokinetic data applying different methodological ideas (see 5.2). Whereas the cut-offs for Norway and the Netherlands are quite similar, for cocaine the pharmacokinetic approach from Denmark leads to different values. Pharmacokinetic profiles for stimulants are analysed and calculated in D 1.1.2b (10).



## 8 Establishing a list of criteria for defining cut-offs

Which criteria should be used for the determination of cut-offs in general?

Experimental and epidemiological results should be interpreted together for the determination of cut-offs.

Recommendations have to be developed concerning substances if the criteria list is not applicable. For instance, pharmacokinetic data may be used when there are not enough scientific results.

### 8.1 Selection of the psychoactive substances for which cut-offs should be determined

#### 8.1.1 Determination of substances, prevalence based

It seems logical to limit the list of drugs for which *per se* limits are given. In many countries, the list is very limited, and contains less than 10 analytes. In Victoria in Australia, the list is limited to 3 substances: THC, methamphetamine and MDMA.

For the selection of analytes, the prevalence in roadside surveys or in injured or killed drivers is a good starting point. The results in the epidemiological studies in DRUID have shown that the prevalence can vary markedly among different countries.

Most countries have started with only illicit drugs, because these have no medical use, and it is less controversial to include them.

It is not a problem to limit the list to a few substances, if the *per se* law is combined with an impairment law, where all other impairing substances are covered. In this scenario, a quick and easy to enforce procedure exists for the most common drugs, and a more elaborate procedure exists for the less frequent cases, including medicinal drugs, combination of drugs, withdrawal, etc. We don't think it is realistic to develop cut-offs for all the existing medicinal and recreational drugs. Moreover for new drugs, it might take some time before the different cut-offs have been established.

#### 8.1.2 Should metabolites be included?

In most cases, *per se* legislation will be limited to the parent drugs and/or active metabolites. Even if the name of '*per se*' or 'zero-tolerance' legislation doesn't imply it, legislators and experts who determine the cut-offs have set them so that the presence of a drug above the cut-off concentration generally means that the person will be impaired. However, in some cases, it is necessary to include metabolites, e.g. when the parent drug is unstable and is metabolised very rapidly, e.g. heroin has a half-life of 3 – 6 minutes and its active metabolite 6-acetylmorphine also has a short half-life and is unstable in blood. In that case, morphine is used, but it is also active.

Cocaine is very unstable in blood that is not preserved with fluoride, so in these cases the inactive metabolite benzoylecgonine is monitored.

As cocaine is very unstable in blood it should be preserved by using sampling tubes with added fluoride. If a country decides to include inactive metabolites as benzoylecgonine the cut-off should be so high that cocaine consumption long time ago (e.g. 12 hours as time for post acute effects) can be excluded.

Some nitrobenzodiazepines like clonazepam, flunitrazepam and nitrazepam are very unstable in blood (17, 18), and their 7-aminometabolites should be measured.

The inclusion or not of metabolites will depend on the choice of matrix, storage conditions and preservatives added in the sampling tubes.

It might also be useful to detect metabolites, not because they are included in the *per se* legislation, but because they increase the level of certainty of the toxicological determination (3).

### **8.1.3 How to deal with combined consumption?**

Much research, including that in DRUID, has shown that the combination of alcohol and drugs, or the combination of more than one drug, increases the accident-risk exponentially. If one applies the cut-offs defined for single use for combined use as well one would accept an increased accident risk.

One could argue that in case more than one drug (including alcohol) is present, the cut-offs don't count, and if the drugs are present above the lower limit of quantitation, the subject can be prosecuted. This has been recommended in WP 6 (19).

### **8.1.4 How to deal with the interval between the accident and the blood sampling?**

In many cases, the delay between the police stop or the accident and the sampling of blood is one hour or more. In the DRUID hospital study, the median time between the accident and sampling was 1.17 hours. But some drugs, like THC, have a very short half-life, particularly in the first hours after smoking. For THC, the half-life of the distribution phase is  $1.4\text{h} \pm 0.1\text{ h}$  (20). If the concentration at the time of the accident was 3.0 ng/mL whole blood, 1.5 hours later it will only be 1.43 ng/mL.

For ethanol, back-calculation is used in many countries, but for drugs, because of the variable pharmacokinetics, back-calculation is very rare.

In order to deal with this, several approaches are possible: one could set the limit lower in order to compensate for the delay between the accident or the police stop and the sampling, or one could allow for back calculation, but this should be standardised among all experts in one jurisdiction. Many toxicologists are reluctant to use back-calculation, considering the many assumptions that have to be made.

### **8.1.5 How to deal with legal prescribed medicine use?**

For this topic a DRUID expert group conducted a workshop with the following results.

A properly prescribed medicine includes right information of the patient by the practitioner. Patients in long-term treatment with psychoactive medicines should not be stigmatised by the need to carry a special "medication passport". Other than with drug users, the responsibility and compliance of patients under long-term treatment usually is high.

It is not reasonable to define cut-off values for patients in long-term treatment. Even high doses may lead to fewer effects. The correlation between dosage and impairment is only intra-individual. There is no clear inter-individual correlation. Dosage effects were only investigated and observed with single users or new users. Hence, an impairment check is an objective way to judge recreational use. Alcohol increases impairment and interacts with many medicines in an unfavourable way. Hence, a separation of drinking, medicine consumption and driving is necessary and the respective information should be part of the physician's consultation.

### **8.1.6 How to deal with tolerance?**

As is the case for alcohol, there are no special cut-offs that take tolerance into account for the cut-off calculation. Cut offs will be defined primarily for occasional (regular) drug users.

## **9 Requirements for analytical methods**

### **9.1 Analytical methods used in DRUID**

Within the epidemiological studies, 13 countries collected whole blood, oral fluid, dried blood spots, plasma or urine from injured drivers in hospital studies and/or from the general driving population during roadside surveys. When the project started, the level of laboratory instrumentation, methodology and expertise varied remarkably between countries. Standardisation, development of laboratory processes, training of staff members and problem solving by study visits to partner laboratories were needed. Requirements were set at the very beginning of the project for ensuring the reliability of the analytical methods used in different laboratories.

Standardisation of data collection and toxicological analysis was very important to allow correct combination of study results. Therefore, several aspects of the toxicological analyses of the oral fluid and whole blood samples like collection and transportation guidelines, target analytes, analytical cut-offs and analytical methods, had to be standardised. This was done in combination with joint epidemiological guidelines for the study protocols (21). Since other matrices such as dried blood spots and urine were only collected in small numbers (and were not used for the epidemiological analysis), no specific guidelines have been developed for these matrices (22).

Twenty-three psychoactive substances were analysed by LC–MS/MS or GC–MS in SIM-mode by all laboratories using the same analytical cut-offs. All laboratories participated in external quality assessment programs (proficiency testing) to further improve comparability of results. The standardisation, requirements and training produced very good results that could be shown by the inter-laboratory proficiency testing results.

The specimens in the epidemiological studies were blood, oral fluid and urine; in the experimental studies serum and plasma. Whole blood was used in the epidemiological studies because legislation in most countries was based on whole blood and transportation was easier since haemolysis was not a concern. Correct collection of plasma was difficult in epidemiological studies because transportation conditions and fluoride preservatives (necessary to prevent cocaine degradation) could cause haemolysis, which makes centrifugation and separation of plasma necessary at the site of collection. Given the facts that sample clean-up of plasma is easier and that collection and transportation of samples is easier to control in experimental studies, plasma is widely used in experimental studies. For practical reasons, most countries collected oral fluid in the epidemiological roadside survey and whole blood in the hospital study.

Literature on the correlation of the analyte concentrations in different body fluids is limited and indicates only a weak or absent correlation between blood and oral fluid concentrations since the ratios between these body fluids show inter individual variation, but can also be time- and dose dependent as well as dependent on collection method of OF (20, 23-26). Literature on pharmacokinetics on the other hand is based only on plasma concentrations.

The main problem for comparison of results was therefore the combination of results obtained from the analysis of different body fluids. This problem was investigated in DRUID by collection and analysis of different sample types collected from the same study persons. The findings from DRUID were used to partially solve these problems.

#### **9.1.1 Consensus on the substrate**

Target analytes were selected based on suspected impairing effects and prevalence of the substances. They were active drugs or their active metabolites except benzoylecgonine, the metabolite of cocaine, because of the stability problem of cocaine.

Project partners had been asked to give their opinion on the relevance of a preselection of analytes. This assessment by the individual partners was both based on (suspected) impairing effects and prevalence in the respective countries. Twenty-three drugs were chosen by at least 9 countries and were included in the 'core list' for which analysis was mandatory in all the 13 countries.

This core list includes ethanol, illicit drugs and their metabolites (amphetamine, MDMA, MDA, MDEA, methamphetamine, cocaine, benzoylecgonine, THC, THC-COOH, 6-acetylmorphine), hypnotics and sedatives (zolpidem, zopiclone, flunitrazepam), anxiolytics (diazepam, alprazolam, nordiazepam, oxazepam, lorazepam), opiates and opiates and medication for substitution treatment (morphine, codeine, methadone), and an antiepileptic agent (clonazepam) (Table 6).

Table 6: Core list of target substances and the cut-off values in whole blood and oral fluid.

Core Substance	Whole blood analytical cut-off (ng/mL)	Saliva analytical cut-off (ng/mL)
Ethanol	0.1 g/L	0.1 g/L
6-acetylmorphine	10	5
Alprazolam	10	1
Amphetamine	20	25
Benzoylecgonine	50	10
Clonazepam	10	1
Cocaine	10	10
Codeine	10	20
Diazepam	20	5
Flunitrazepam	2	1
Lorazepam	10	1
MDA	20	25
MDEA	20	25
MDMA	20	25
Methadone	10	20
Methamphetamine	20	25
Morphine	10	20
Nordiazepam	20	1
Oxazepam	50	5
THC	1	1
THCCOOH	5	NR
Zolpidem	20	10
Zopiclone	10	10

In order to include more classes of medicinal drugs, all countries were asked to individually add at least three more analytes. As a result, in total 28 additional drugs were included as 'national drugs' (Table 7). The extra substances belong to the drug groups that were already present in the core list, but also include antihistamines, antidepressants (both SSRI's, non-selective monoamine reuptake inhibitors and other antidepressants) and antipsychotics: 7-aminoclonazepam (metabolite of clonazepam, 12 countries), 7-amino-flunitrazepam (metabolite of flunitrazepam, 11 countries), tramadol (8 countries), amitriptyline and nitrazepam (6 countries), buprenorphine (5 countries), bromazepam (4 countries), diphenhydramine and midazolam (3 countries), 11-OH-THC, carisoprodol, (es)citalopram, meprobamate, mirtazapine, temazepam, trazodone (2 countries), carbamazepine, chlordiazepoxide, ecgonine methyl ester (metabolite or break-down product of cocaine), fluoxetine, haloperidol, imipramine, ketamine, melperone, olanzapine, phenazepam, venlafaxine and zaleplon (1 country).

Table 7: The list of additional substances measured in some countries.

Substance	Whole blood analytical cut-off (ng/mL)	Saliva analytical cut-off (ng/mL)
11-OH THC	1	NA
7-Aminoclonazepam	10	1
7-Aminoflunitrazepam	2	1
7-aminonitrazepam	10	1

Substance	Whole blood analytical cut-off (ng/mL)	Saliva analytical cut-off (ng/mL)
Acetamidoclonazepam	1	1
Acetamidonitrazepam	1	1
Amitriptyline	10	10
Bromazepam	20	5
Brotizolam	1	1
Buprenorphine	1	1
Carbamazepine	NA	10
Carisoprodol	500	50
Chlordiazepoxide	20	10
Citalopram	5	5
Clobazam	5	5
Demoxepam	10	10
Desalkylflurazepam	2	2
Desmethylchlordiazepoxide	8	8
Desmethylclobazam	5	5
Desmethylflunitrazepam	1	1
Diphenhydramine	NA	10
Fluoxetine	10	5
Flurazepam	2	1
Imipramine	NA	10
Ketamine	20	20
Levomepromazine	NA	10
Lormetazepam	1	1
Meprobamate	2000	1200
Methylecgonine	5	5
Midazolam	10	2
Mirtazapine	5	5
Nitrazepam	1	2
OH-alprazolam	1	1
OH-ethylflurazepam	2	2
OH-midazolam	1	1
OH-triazolam	1	1
Temazepam	20	10
Tramadol	50	50
Trazodone	10	5
Triazolam	1	1

### 9.1.2 Standardisation of collection and methods

Collection and transportation conditions were selected so that they should ideally ensure stability of all compounds in the different sample types. Literature on this issue is limited for most analytes. It was however clear that within the 'core list', cocaine is the most unstable compound during transportation. Since cocaine degradation in whole blood is 22.9% per day at 20 °C and only 4.5% per day at 4 °C, it was critical that samples were transported at 2–8 °C (27). Transportation was not allowed to last more than 48 h. After transportation, samples had to be stored at –20 °C prior to analysis. Whole blood (5–10 mL) was collected using glass Vacutainer-type tubes containing sodium fluoride and potassium oxalate.

The type of device used for oral fluid collection was critical since this strongly influences the concentrations present (28). The device had to allow fast and easy collection, ensure good stability and recovery of illicit and medicinal drugs and provide enough samples for toxicological analysis. Ten devices were compared on these criteria. Finally, the Statsure™ saliva sampler (Statsure Diagnostics Sys-

tems, Framingham, MA, USA) was chosen. All tested substances had a recovery >80% and good results for stability over 28 days (29).

### 9.1.3 Analytical methods

Since only a small volume of sample was available, all laboratories had to develop methods for simultaneous detection of the target analytes. All laboratories agreed to use either LC–MS/MS or GC–MS in SIM-mode. The choice of technique for the determination of ethanol was open, quantitative breathanalyser results were accepted for use as well. Several analytical methods used for the analysis have been published or presented at international meetings (30-36).

### 9.1.4 Quality assurance

The laboratories implemented planned and systematic activities within their quality system, to provide adequate confidence that the results fulfilled the requirements for quality set in DRUID. The laboratories improved their quality control, the overall system of laboratory procedures and processes that controlled the quality of the laboratory's analytical results. Methods used in DRUID had to be developed, evaluated and tested to ensure that they produce valid results at the agreed levels. The methods had to be validated in order to confirm that the requirements in DRUID are fulfilled. Performance characteristics of the analytical methods were evaluated, including e.g. the ability to get the true results (accuracy /bias), linearity, limit of detection (LOD), lower limit of quantitation (LLOQ), recovery, precision/ repeatability, reproducibility, ruggedness, specificity (selectivity), matrix effect and uncertainty of measurement (4).

**The analytical cut-off concentrations** (or thresholds) were decided in DRUID and all the laboratories had to have methods sensitive enough to reach the set cut-offs. A cut-off is the agreed concentration of a drug in a specimen that is used to determine whether the specimen is considered positive or negative. The cut-off had to be higher than the limit of detection. (4).

The ability to quantify was evaluated by LOQ. The limits of quantitation should not have been higher than the established cut-off values in DRUID.

Analytical cut-offs were established for the core list based on those used in ROSITA-2 (37), SAMHSA cut-off values (38) for oral fluid and recommendations from an expert meeting in Talloires (39) (Table 6). Because of practical and legal considerations, different sample types were used: whole blood, serum/plasma and oral fluid and cut-offs were determined for oral fluid, serum/plasma and blood.

### 9.1.5 Proficiency testing

Proficiency testing schemes were used to monitor and compare the participating laboratories' performance against that of other partners' laboratories in producing equivalent data. Even with the tight standardisation efforts, differences between laboratories may still occur. These were detected by proficiency testing (PT) for both oral fluid and whole blood, which were organised twice per year. In recent years several PT programs for drugs of abuse in oral fluid have started (40-42). For DRUID, a new program was specifically designed and organised by RTI International (Research Triangle Park, NC, USA): five samples of 1.5 mL of synthetic oral fluid (spiked with the 'core list' analytes) were distributed to all DRUID laboratories every 6 months. Arvecon GmbH (Walldorf, Germany) organised a specific whole blood PT scheme. When problems in proficiency testing were detected, corrective actions were made and the proficiency testing results improved. The sample analysis started only after the proficiency testing results showed that the substances were detected and quantitated correctly. All measures were taken to reduce error probability. Some proficiency testing results are presented in Figure 8. The results of the oral fluid proficiency testing have been published (43).

Analyte	Sample	Number	Successful participants	Spiked value	Target value	SD <sub>Horwitz</sub> <sup>1</sup>	VC <sub>Horwitz</sub> <sup>2</sup>	Accepted range
6-MAM	A	25	23 (92%)	100,00	113,30	25,20	0,22	62,90 - 163,70
Alprazolam	A	24	21 (88%)	30,00	28,90	7,90	0,27	13,10 - 44,70
Amphetamine	A	26	25 (96%)	550,00	534,90	94,00	0,18	346,90 - 722,90
Benzoylcegonine	A	27	27 (100%)	425,0	362,50	67,60	0,19	227,30 - 497,70
Clonazepam	B	25	23 (92%)	25,00	25,10	7,00	0,28	11,10 - 39,10
Cocaine	A	27	26 (96%)	50,00	46,10	11,70	0,25	22,70 - 69,50
Codeine	B	27	24 (89%)	250,00	251,80	49,60	0,20	152,60 - 351,00
Diazepam	B	25	24 (96%)	350,00	359,30	67,10	0,19	225,10 - 493,50
Flunitrazepam	B	26	25 (96%)	20,00	20,00	5,80	0,29	8,40 - 31,60
Lorazepam	B	24	17 (71%)	55,00	41,90	10,80	0,26	20,30 - 63,50
MDA	A	26	25 (96%)	25,00	24,00	6,70	0,28	10,60 - 37,40
MDMA	A	25	24 (96%)	250,00	241,90	47,90	0,20	146,10 - 337,70
Methadone	A	26	26 (100%)	175,00	162,40	34,20	0,21	94,00 - 230,80
Methamphetamine	A	26	23 (88%)	450,00	415,80	75,90	0,18	264,00 - 567,60
Morphine	A	27	26 (96%)	150,00	141,60	30,40	0,21	80,80 - 202,40
Nordiazepam	B	26	25 (96%)	450,00	395,40	72,80	0,18	249,80 - 541,00
Oxazepam	A	25	22 (88%)	450,00	440,70	79,80	0,18	281,10 - 600,30
THC	A	27	24 (89%)	18,00	16,20	4,80	0,30	6,60 - 25,80
THCCOOH	A	26	24 (92%)	95,00	88,50	20,40	0,23	47,70 - 129,30
Zolpidem	B	24	20 (83%)	120,00	100,40	22,70	0,23	55,00 - 145,80
Zopiclone	B	21	15 (71%)	280,00	301,20	57,70	0,19	185,80 - 416,60

<sup>1</sup> standard deviation according to Horwitz <sup>2</sup> variation coefficient (Horwitz)

Figure 7: Results of the proficiency testing in blood, 1st round of 2010

Analyte	DRUID cutoff (ng/mL)	Sample concentration range (ng/mL)	Number of sample challenges	Sens	Spec	CV			Outliers <sup>5</sup>
						Average	Min	Max	
6-acetylmorphine	5	8–15	4	93.9%	100%	27.4%	21.7%	34.8%	
Alprazolam	1	2–6	4	92.7%	100%	26.0%	17.4%	41.2%	2
Amphetamine	25	50–100	4	92.7%	100%	22.9%	12.3%	42.3%	
Benzoylcegonine	10	20–80	5	98.1%	100%	31.2%	25.5%	36.4%	2
Clonazepam	1	2–2	2	100%	100%	27.4%	21.3%	33.5%	
Cocaine	10	20–80	6	96.7%	99.3%	19.1%	13.1%	24.5%	
Codeine	20	25–60	3	100%	100%	35.3%	32.1%	41.1%	
Diazepam	5	10–15	2	100%	99.5%	22.2%	17.2%	27.2%	
Ethanol	0.1 g/L	0.2–0.8 g/L	8	100%	100%	11.2%	6.5%	18.9%	2
Flunitrazepam	1	2–6	2	81.8%	99.5%	28.8%	19.7%	37.9%	
Lorazepam	1	2–3	2	90.9%	100%	20.4%	18.1%	22.7%	1
MDMA	25	50–75	2	100%	100%	35.9%	31.2%	40.7%	
MDA	25	75–75	2	100%	100%	30.8%	26.9%	34.7%	
MDEA	25	75–75	2	100%	100%	28.9%	27.8%	30.0%	1
Methadone	20	30–40	2	100%	99.5%	18.5%	18.3%	18.7%	
Methamphetamine	25	40–75	3	94.7%	100%	28.7%	28.1%	29.0%	1
Morphine	20	25–60	4	100%	100%	28.1%	13.5%	46.6%	1
Nordiazepam	1	2–5	2	95.5%	99.5%	18.3%	16.2%	20.4%	
Oxazepam	5	10–10	2	94.7%	99.5%	27.4%	17.0%	37.7%	
THC	1	3–10	8	93.9%	100%	25.7%	13.7%	35.2%	1
Zolpidem	10	15–40	3	93.3%	100%	22.2%	16.2%	32.6%	
Zopiclone	10	15–40	6	81.7%	99.3%	34.5%	15.2%	65.1%	2

<sup>5</sup>Outlier defined as value with more than 100% deviation from mean.

Figure 8: Oral fluid PT: number of samples spiked with target analytes, concentration ranges and DRUID cut-offs. Sensitivity and specificity, minimum, maximum and average coefficients of variation (43).

## 9.2 Measurement uncertainty

Every measurement is subject to some uncertainty. Measurement uncertainties can come from the measuring instrument, from the item being measured, from the environment, from the operator, and from other sources. Such uncertainties can be estimated using statistical analysis of a set of measurements, and using other kinds of information about the measurement process. There are established rules for how to calculate an overall estimate of uncertainty from these individual pieces of information. The use of good practice – such as traceable calibration, careful calculation, good record keeping, and checking – can reduce measurement uncertainties (44).

The definition of the term uncertainty (of measurement) is: "A parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand" (45).

There are different types of **precision**. The precision is a measure for the size of random errors. It measures the dispersion around the mean result and, therefore, it requires the calculation of the standard deviation of the measurement results. Precision can be determined at several levels. **Repeatability** is measured under repeatability conditions, meaning that the operator, the instrument and the laboratory are the same, and the time interval is kept short. These are the most favourable conditions possible and they yield the best precision, (i.e., the smallest standard deviation).

**Reproducibility** is defined as measured under "conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment." It takes into account many more sources of variation than the repeatability does. These are the worst precision conditions that can occur when studying the precision of a method. It can be determined only with inter-laboratory method performance studies, colloquially known as collaborative trials. (46)

Intermediate situations occur and give rise to an **intermediate precision**. They take into account more within-laboratory variations than when the precision is measured under repeatability conditions, such as the additional variation due to the measurements being performed over a longer period of time. The intermediate precision can then be seen as a measure of long-term precision in a given laboratory.

A fourth somewhat different level is the determination of **robustness** (sometimes also called ruggedness). It measures to what extent a procedure is affected by small, deliberate variations introduced in the procedure. If one or more of these variations are found to be responsible for a significant difference in the results, the procedure must be adapted and more strictly controlled. If not, the method is considered robust, but the variations still lead to less precise measurements and robustness can therefore be seen as a measure of the intermediate precision or the reproducibility that might be expected.

ISO uses the symbol  $r$  for repeatability and  $R$  for reproducibility. Repeatability and reproducibility are measured as the repeatability standard deviation,  $s_r$ , and the reproducibility standard deviation,  $s_R$ . For the intermediate precision ISO proposes the symbol  $I_{( )}$  with additional symbols inside the parentheses referring to the intermediate precision conditions. In this way  $s_{(TO)}$ , for example, means that the intermediate precision includes variability due to the time elapsed between measurements as well as due to the operator.

The Horwitz curve gives an indication of the precision to be expected of a newly developed method as a function of the concentration of the analyte. It is named after W. Horwitz, a respected statistician, now retired from the Food and Drug Administration (FDA). Horwitz et al. initially examined results of a few thousand interlaboratory collaborative studies on various commodities ranging in concentration from a few percent (salt in foods) to the ppb (ng/g) level (aflatoxin M1 in foods) but also including studies on, for example, drug formulations, antibiotics in feeds and pesticide residues. They concluded that the predicted  $RSD_R$  (%) as a function of concentration is approximated by the following relationship:

$$\text{Predicted } RSD_R \% = \sigma_H = 2(1 - 0.5 \log_{10} C)$$

where  $C$  is the concentration expressed as a dimensionless fraction (for example for a concentration of  $1\mu\text{g/g}$ ,  $C = 10^{-6} \text{ g/g}$ ). In this context the predicted  $RSD_R$  % is sometimes also written as  $\sigma_H$ , where the  $H$  stands for Horwitz. Equation 1 still holds for the 10000 interlaboratory studies that have been evaluated up to now. It states that  $\sigma_H$  approximately doubles for every 100-fold decrease in concentration, starting at 2% for  $C = 1$ . The graphical representation of Equation 1 is referred to as the Horwitz curve and is shown in Figure 9. In

Table 8 some  $\sigma_H$  are calculated for some DRUID cut-offs for some analytes.



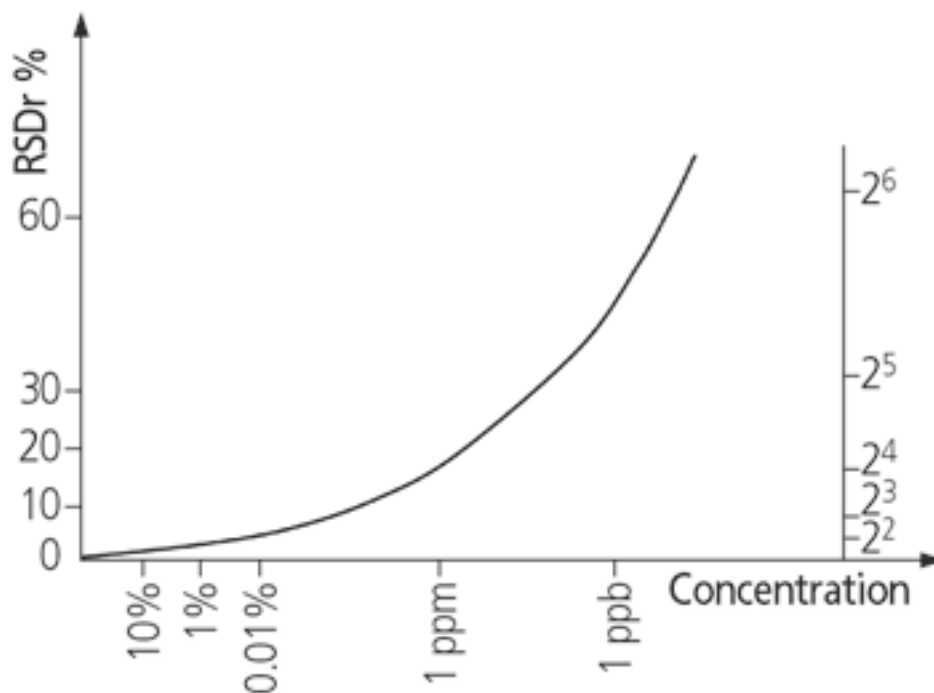


Figure 9: Relative reproducibility standard deviation  $RSD_R$  as a function of concentration (46).

Table 8: Predicted relative reproducibility standard deviation for some DRUID analytes (in%) at the cut-off, calculated with the Horwitz equation.

Analyte	DRUID cut-off	$\sigma_H$
Ethanol	0.1 g/L	8%
THC	1 ng/mL	45%
Morphine	10 ng/mL	32%
Oxazepam	50 ng/mL	25%
THCCOOH	5 ng/mL	35%

One of the more remarkable aspects of the Horwitz curve is its generality. It probably appears strange to the analyst that the reproducibility and the repeatability do not depend on the analytical method used. One of the reasons is that the method being investigated is studied intensively before an interlaboratory study is undertaken, so that as many sources of variation as possible are kept under control by including the necessary specifications in the procedure to be followed. Thus, methods difficult to control or operating too close to detection limits, etc, will be not be subjected to interlaboratory studies.

Another interesting result of the Horwitz study is that the corresponding repeatability measure ( $RSD_r$ ) is generally one-half to two-thirds of the reproducibility measure  $RSD_R$ . These repeatability figures are based on the repeatability of the laboratories participating in the interlaboratory studies that formed the basis of Horwitz's study. They are determined under the strict rules of ISO or the AOAC/IUPAC protocols for such studies. Repeatabilities determined by individual laboratories outside such studies often tend to underestimate the variation and therefore yield too optimistic results.

#### Uncertainty and the Horwitz Curve.

The idea is to create an interval around the analytical result such that there is a 95% certainty that the true value is encompassed in it. This interval, known as the expanded uncertainty, is obtained as result  $\pm 2sR$ . The value of  $\sigma_H$  derived from the Horwitz curve can be used as a best guess when  $sR$  is not known (yet). When a given maximum level of uncertainty is required for some application, for which the analytical method still has to be developed, the analyst can use  $\sigma_H$  to evaluate the probability that the method will prove fit for its purpose.

Based on this information, the measurement uncertainty is approximately 30%-45% in the  $\mu\text{g/L}$  range. The measurement uncertainty that is used in Switzerland is 30%. In Denmark a measurement uncertainty of 50% is used before a positive result is reported.

## Part 2: Analytical findings in DRUID

### 10 Toxicological and analytical findings in DRUID

#### 10.1 Determining the oral fluid to whole blood ratios for the DRUID core substances

##### 10.1.1 Introduction

In the epidemiological studies of the DRUID project (WP2) both oral fluid (OF) and whole blood samples have been collected due to practical and legal considerations. Blood and urine are the traditionally used sample matrices in drug testing, but recently the use of OF has increased due to its simple and noninvasive collection. Blood is used especially in driving under the influence (DUI) cases when proof of very recent drug use or possible impairment is needed. Detection times of drugs in OF better reflect the ones in blood than urine (47), which is mainly used to detect previously occurred or chronic drug use. In order to compare results from two different sample matrices the relationship of the drug concentrations between the two has to be determined (22). There have been studies on the subject where the connection between the concentrations has usually been determined by calculating the OF to whole blood (OF/B) or OF to plasma (OF/P) ratios for different drugs, but the results between the studies seem to differ quite a lot, and even within a single study the variation in the results can be more than 100% RSD (48). The concentration of drugs in OF depends on several variables, including the drug's pKa, lipid solubility, molecular weight and plasma-protein-binding, and also on the dose and time of drug intake. Compared to blood, there is a delay for some drugs to be detectable in OF. In addition, different sample collection methods may result in different concentrations, and also the inter-individual differences in OF pH and salivary flow can affect the OF drug concentrations (49, 50). This makes it very complicated, if not impossible, to find a certain universal conversion factor between whole blood and OF drug concentrations.

The aim of the study was to determine OF/B ratios for DRUID core substances in order to compare the analysis results of OF and whole blood samples collected in the epidemiological studies of the DRUID project.

##### 10.1.2 Sample collection and analysis

Paired OF and whole blood samples were collected in Belgium, Finland, Italy and Norway from drivers involved in traffic accidents, drivers suspected of DUI and random drivers stopped at roadside. OF sampling was done with the StatSure SalivaSampler™ (StatSure Diagnostic Systems, Inc., Framingham, MA, USA). The time difference between the collection of OF and whole blood sample was less than 30 minutes for all paired samples. The quantitative analysis of samples was performed with either liquid chromatography-tandem mass spectrometry or gas chromatography-mass spectrometry (or tandem mass spectrometry). All analytical methods were validated and external proficiency testing rounds were organised to assure the accuracy of the results.

##### 10.1.3 Data analysis

OF/B ratios were calculated for each substance by dividing the OF concentration with the whole blood concentration, and zero OF/B ratios were excluded (i.e. cases where OF was negative and blood positive). Outlier OF/B ratios were identified by using box and whisker plots and the cases outside the outer fences were excluded as extreme outliers.

After the exclusion of outliers, the following values were calculated for all substances: mean (with 95% confidence intervals) and median OF/B, standard deviation and the range of OF/B ratios. For these

calculations, only substances that had 3 or more cases that were positive in both matrices were included.

#### 10.1.4 Results and discussion

The calculated OF/B ratios and their descriptive statistics are presented in Table 9. For 6-AM, flunitrazepam, MDA, MDEA, and MDMA the number of cases for the OF/B ratio calculations was less than 6, thus the results of these substances should be interpreted with caution. Also, even for substances that had more than 6 cases the range of the OF/B ratios is very wide and the large standard deviations of the mean OF/B ratios imply that the determination of an accurate conversion factor is very difficult.

Correlation of the concentrations between the two matrices was also studied by drawing the scatter plots of whole blood vs. OF concentration. Figure 10 and Figure 11 show the linear correlations for amphetamine and THC, respectively. As the figures show, the correlation for amphetamine is quite good, with  $R^2 = 0.868$ , whereas there does not seem to be any correlation for THC, which has an  $R^2$  of only 0.0003. Also, the number of cases with the other matrix negative (64%) is very high for THC. Other substances that have the other matrix negative in more than 50% of cases are 6-AM, cocaine, codeine, lorazepam, MDMA, morphine, oxazepam and zopiclone.

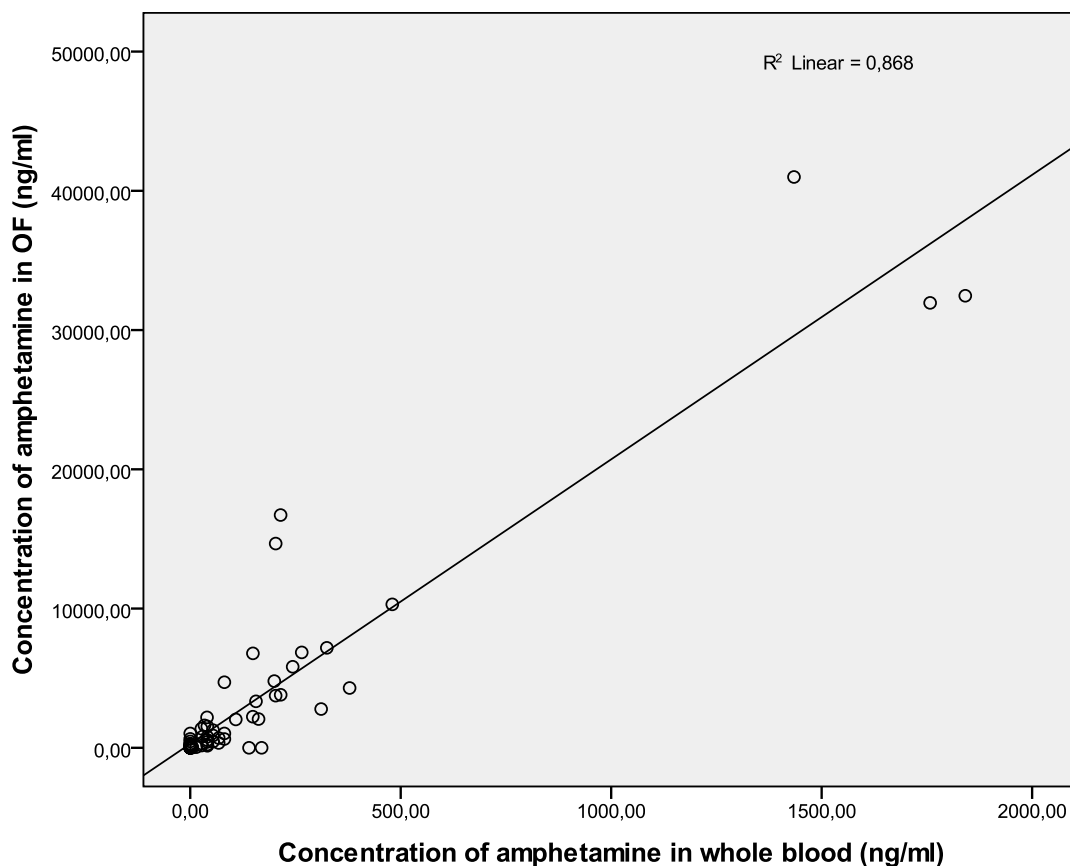


Figure 10: Correlation of amphetamine concentrations between whole blood and oral fluid.

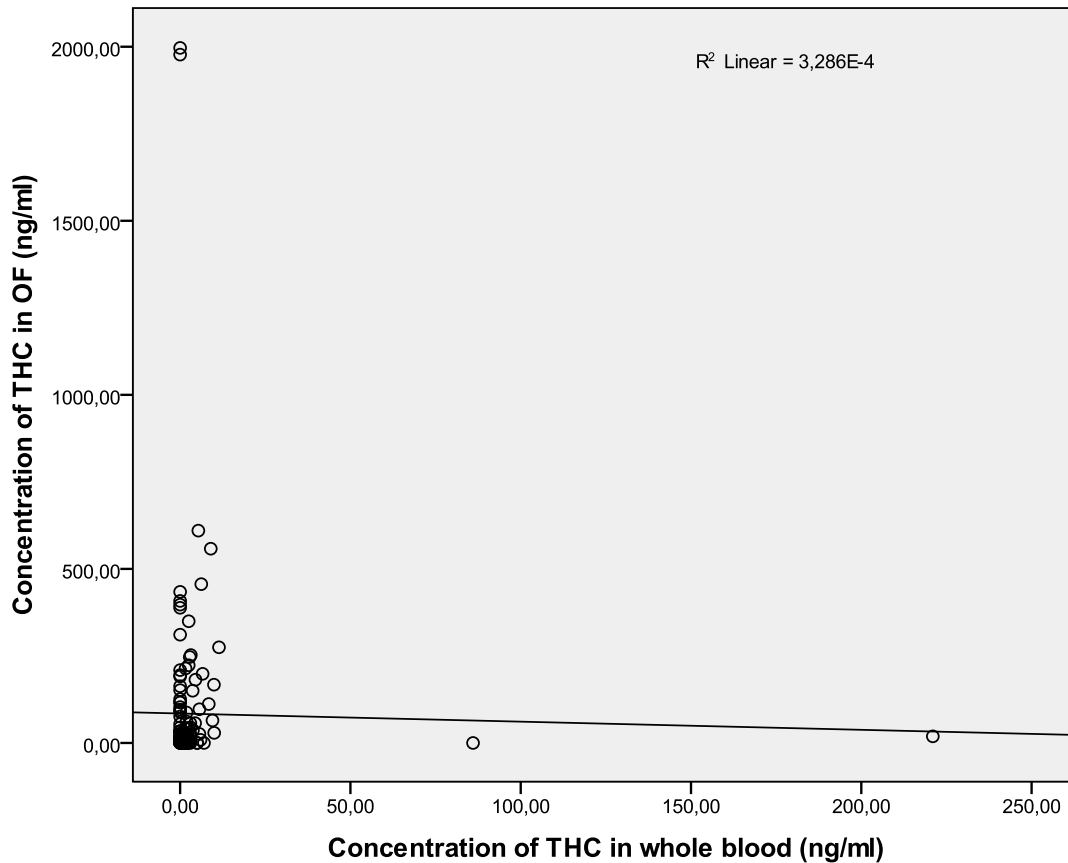


Figure 11: Correlation of THC concentrations between whole blood and oral fluid.

#### 10.1.4.1 An example of the problem of determining which OF/B ratio to use as a 'conversion factor'

If one would calculate a blood concentration for nordiazepam (that has a fairly good correlation between OF and whole blood,  $R^2 = 0.72$ ), when the concentration in OF is 20 ng/ml, one would get a range of 327-418 ng/ml by using the 95% confidence intervals for mean. By dividing the OF concentration with the mean OF/B the whole blood concentration would be 368 ng/ml and by using the median value it would be 420 ng/ml. The challenge of deciding which OF/B value to use in the calculations will be covered in the following section of the report, titled Estimation of equivalent cut-off thresholds in blood and oral fluid

Table 9: Calculated OF/B ratios and their descriptive statistics for each DRUID core substance and three additional substances.

Substance	N (total cases with positive findings)	N (other matrix negative or below LOQ)	N (OF/B outliers*)	N (OF/B for calculations)	mean OF/B (95% confidence intervals)	SD for mean OF/B	median OF/B	min – max OF/B (outliers removed)
6-AM	22	18	0	4	61.88 (-93.24-217.01)	97.49	15.70	8.29-207.9
alprazolam	91	20	7	64	0.3499 (0.3009-0.3988)	0.1959	0.3310	0.03-1.06
amphetamine	73	29	2	42	22.57 (16.82-28.32)	18.46	17.95	3.27-77.86
benzoyllecgonine	84	41	5	38	2.821 (1.833-3.808)	3.005	1.578	0.18-13.34
clonazepam	52	14	5	33	0.1949 (0.1397-0.2501)	0.1557	0.1509	0.03-0.73
cocaine	84	59	3	22	20.53 (13.34-27.72)	16.21	17.05	1.19-63.26
codeine	93	48	5	40	6.959 (5.077-8.840)	5.883	4.698	0.17-23.85
diazepam	81	37	5	39	0.0475 (0.0355-0.0594)	0.0368	0.0347	0.0005-0.20
flunitrazepam	1	0	0	1	0.1905			
lorazepam	35	22	0	13	0.1086 (0.0789-0.1384)	0.0492	0.0952	0.06-0.23
MDA	5	2	0	3	11.22 (5.35-17.10)	2.365	11.67	8.67-13.33
MDEA	0			0				
MDMA	15	11	0	4	13.56 (-1.87-29.00)	9.698	11.96	3.58-26.76
methadone	16	5	1	10	2.810 (1.069-4.550)	2.433	1.5009	0.69-7.12
methamphetamine	50	14	0	36	28.88 (21.67-36.10)	21.33	20.68	5.61-85.90
morphine	75	39	2	34	9.498 (6.360-12.637)	8.995	5.917	0.60-36.73
nordiazepam	117	44	4	69	0.0544 (0.0478-0.0611)	0.0278	0.0476	0.01-0.12
oxazepam	53	31	2	20	0.1709 (0.1064-0.2354)	0.1378	0.1219	0.03-0.51
THC	162	103	4	55	26.19 (17.18-35.19)	33.31	14.12	0.09-138.8
zolpidem	26	5	1	20	0.3617 (0.2622-0.4611)	0.2124	0.2733	0.05-0.83
zopiclone	17	10	1	6	2.519 (1.262-3.776)	1.198	2.400	1.27-4.72
<i>Additional substances</i>								
tramadol	26	4	0	22	12.93 (8.54-17.32)	9.91	11.08	1.40-33.50
7-amino-clonazepam	12	5	1	6	0.3124 (0.1971-0.4278)	0.1099	0.2778	0.20-0.49
7-amino-flunitrazepam	2	1	0	1	0.1176			

\* outliers are the cases for which the calculated OF/B ratio was an extreme outlier according to the box and whisker plot

## 10.2 Conversion factors for whole blood and plasma

Different DUID legislations use different matrices. In Europe, Germany uses serum, Belgium uses plasma and most of the other countries use whole blood (see Table 2). For this reason, whole blood was used as the common matrix in the epidemiological studies in DRUID.

Blood is a complex mixture that contains solubilised proteins, dissolved fats, solids and suspended cells. Serum or plasma is traditionally used in clinical settings because blood affords advanced handling in the laboratory procedures. Traditionally, pharmacokinetic studies have been performed on plasma or serum, while most post-mortem results are expressed in whole blood. This is because after death the red blood cells lyse. Hence, separation of red blood cells from post-mortem blood is usually not possible, and its composition may differ from a blood sample obtained from a living person. Therefore, literature data of serum/plasma concentrations cannot be absolutely used to classify the concentrations determined from post-mortem blood. Moreover, the concentration and characteristics of cellular and extra cellular quantities differ from the *ante mortem* state. The water content and pH of a post-mortem blood sample may also differ significantly from physiological ranges. The water content of post-mortem blood was observed to range from 60 to 90%. Immediately after death, there is a sharp decrease in pH up to 5.5, which again slowly increases during the post-mortem interval due to the break down of protein. Blood samples from the post-mortem examination are often haemolysed, putrefied, and may be quite inhomogeneous mixtures. Blood collected at autopsy may be clotted or completely fluid or partly clotted and partly fluid. Large numbers of red blood cells are entrapped by clots, therefore toxicological analysis of such material will influence the detected level of any drug that is unevenly distributed between serum or plasma and cellular constituents of blood.

Despite these limitations, it is useful to have conversion factors to calculate whole blood concentrations to plasma concentration and vice-versa. There is some literature on the subject, but surprisingly few studies have been performed.

Like all biological variables, these ratios are not constant, and there is some biological variation. Moreover, as the relative proportion of plasma and red blood cells varies, the ratio might also depend on the haematocrit (the percentage of blood volume that is occupied by red blood cells. It is normally about 45% for men and 40% for women). For some drugs, varying blood to plasma ratios have been observed between individuals. In patients, chlorpromazine erythrocyte concentrations tended to correlate with plasma concentrations, but the erythrocyte/plasma concentration ratio varied from 0.61 to 2.00 between patients. Ratios may not only vary between drugs but may also differ between a particular drug and corresponding metabolites. The blood to plasma ratio of morphine was unaffected by variations in haematocrit and water content, whereas the corresponding ratios for morphine-3- and morphine-6-glucuronide were strongly influenced. The distribution of cannabinoids between blood and serum determined on specimens from living persons was within a closer range than the values obtained from post-mortem samples. Applying blood to serum/plasma ratios from literature data to post-mortem samples, it should be kept in mind that during lifetime an uneven blood to plasma ratio is maintained by active processes, which may decay after death. Generally, the differences observed between blood and plasma are considered to be of minor importance compared to the changes in concentration that may occur prior to sampling. (51)

Many drugs, particularly the benzodiazepines, are highly bound to plasma protein, which has implications when forensic toxicology results are compared with therapeutic ranges based on analysis of plasma or serum. For protein-bound drugs such as diazepam, women will have a somewhat lower concentration in whole blood than men owing to gender-related differences in haematocrit. Likewise, any medical conditions that result in an abnormal red cell volume such as anaemia or polycythaemia also deserve consideration when the concentration of drugs are compared and contrasted between plasma and whole blood. The small interindividual variations in blood haematocrit have not until now been considered when forensic toxicology results are compared with therapeutic ranges based on therapeutic drug monitoring data (52).

What should be considered when the concentration of a drug in whole blood is compared with the therapeutic range in plasma or serum and a decision made whether the person has overdosed? A good starting point are scientific papers reporting the maximal concentration ( $C_{max}$ ) after a single

therapeutic dose and the concentration at steady state ( $C_{ss}$ ) after long-term therapy. Whenever possible, pharmacokinetic parameters should be considered in relation to age, gender, ethnicity, obesity, and any known liver or kidney dysfunction. External factors such as consumption of alcohol, alcohol-induced liver disease, smoking, use of contraceptive steroids, pregnancy, and concomitant use of other drugs might influence  $C_{max}$  or  $C_{ss}$  under some circumstances. Pharmaceutical aspects such as the dosage form, whether tablet, syrup, or sustained-release product, as well as route of administration can have a large impact on  $C_{max}$  for the same dose of drug administered.

In general, the concentration of a drug in plasma or serum is higher than in whole blood, especially for protein-bound drugs with some obvious exceptions such as the antimalaria drug chloroquine, which is preferentially concentrated in the erythrocytes. When the concentration of a drug in whole blood (forensic cases) is compared with the concentration in plasma or serum (therapeutic drug monitoring cases), this gives an advantage to the suspect when one has to decide whether the analytic result is suggestive of an overdose of the medication in question.

The blood/plasma ratios for the core drugs from the literature are given in Table 10.

Table 10: Blood/plasma ratios obtained from the literature.

Drug	Blood/plasma ratio	Source
Alcohol	0.83 0.74 – 0.90	(53) (51)
Amphetamine	0.6-1.1; 1.0 0.6 at 0.5 mg/L 1.0 at 5.0 mg/L	(53, 54)
Methamphetamine	0.65	(55)
MDMA	1.26	(56)
MDEA	No data	
MDA	1.27	(56)
Cocaine	1.0	(51)
Benzoylcegonine	No data	
THC	0.55, 0.66	(53-55)
11-OH-THC	0.62	(51)
THCCOOH	0.57 – 0.58 0.45 – 0.71	(51) (57)
6-acetylmorphine	No data	
Morphine	1.02	(51)
Codeine	0.87	(51)
Tramadol	No data	
Methadone	0.75-1.0	(55)
Diazepam	0.55-0.70	(51, 53, 55)
Nordiazepam	0.59	(53, 55)
Alprazolam	0.8; 0.625	(51, 53)
Oxazepam	0.9-1.0	(51, 58)
Flunitrazepam	0.75	(59)
Zopiclone	1.0	(51)
Zolpidem	No data	
Trazodone	0.64	
Clonazepam	0.65	(53)
Lorazepam	No data	
GHB	1.20	(59)

Based on the analysis at the University of Heidelberg for some experimental studies in DRUID, whole blood/serum ratios were determined for some opioids, THC, OH-THC and THC-COOH, dexamphetamine, risperidone and its hydroxyl metabolite, alprazolam and zopiclone.



Table 11: Whole blood/serum ratio based on DRUID results: minimum and maximum value, median ratio and ratio based on Bland and Altman calculation.

Drug	n	minimum	maximum	median	Bland & Altman
Buprenorphine	5	0.50	0.63	0.54	0.55 (0.45-0.65)
Norbuprenorphine	5	0.95	1.03	0.97	0.98 (0.91-1.05)
Fentanyl	13	0.62	1.02	0.91	0.87 (0.63-1.11)
Norfentanyl	13	1.03	1.55	1.22	1.26 (0.96-1.46)
Hydromorphone	15	0.91	1.22	1.00	1.04 (0.87-1.20)
Morphine	6	0.96	1.07	1.02	1.02 (0.95-1.09)
Oxycodone	12	1.29	1.76	1.49	1.48 (1.24-1.72)
Noroxycodone	12	1.28	2.09	1.81	1.73 (1.27-2.19)
Tramadol	2	1.15, 1.24			
N-desmethyltramadol	2	1.21, 1.29			
O-desmethyltramadol	2	0.94, 1.40			
THC	165	0.33	0.94	0.62	
OH-THC	173	0.30	0.82	0.60	
THC-COOH	197	0.41	0.83	0.59	
Dexamphetamine	29	0.65	1.14	0.89	
Risperidone	10	0.56	0.71	0.66	
9-OH-risperidone	14	0.61	0.91	0.73	
Alprazolam	28	0.69	0.93	0.82	
Zopiclone	45	0.66	1.29	0,86	

### 10.3 Estimation of equivalent cut-off thresholds in blood and oral fluid

There are large variations in the OF/B ratios for a substance between individuals (see section 10.1). Standard deviations in OF/B ratios of more than 50% are common. Therefore, conversion factors cannot be used for accurate estimation of drug concentrations in blood based on drug concentrations in oral fluid.

In a cohort of drug users, the prevalence of drug concentrations above a given cut-off in oral fluid will reflect drug use in that cohort. The drug prevalence in oral fluid and blood will be equal if the cut-off concentrations are equivalent and if the cohorts are large. Equivalent cut-off concentrations in blood and oral fluid also imply that both specimens will, on average, be positive for a drug for the same length of time following the intake of a single drug dose.

It has previously been found that approximately equivalent cut-off concentrations in blood and oral fluid for amphetamine and THC may be estimated by multiplying the cut-off concentration in blood with the average or median OF/B ratio or with the regression coefficient (60). Alternative methods are prevalence regression using aggregated population data, which in some cases may give more accurate estimations (61) or a mathematical simulation, which is a more challenging procedure (60). The latter procedure is only applicable for large populations (e.g. more than 75 individuals).

The aim of this study was to estimate equivalent drug cut-off concentrations in blood and oral fluid collected with Statsure Saliva sampler for estimating the prevalence of drug use in a cohort or population.

#### 10.3.1 Sample collection and analysis

See section 10.1.2

### 10.3.2 Procedures for estimating equivalent cut-off concentrations

#### 10.3.2.1 Identification of outliers

Outliers regarding OF/B ratios were identified using box and whisker plots, defining values outside the outer fences as outliers.

#### 10.3.2.2 Using average or median OF/B ratios

The formula for calculation of equivalent cut-off concentrations were:  $C_{OF} = C_B \times F$ , where  $C_{OF}$  = drug concentration in oral fluid,  $C_B$  = drug concentration in blood, and  $F$  = either the average or median OF/B ratio.

#### 10.3.2.3 Using prevalence regression

Mathematical models describing the regression between concentration percentiles in oral fluid as a function of the corresponding concentration percentiles in blood were determined using paired samples being positive for the studied substance in both oral fluid and blood. The drug concentrations in oral fluid corresponding to selected percentiles, e.g. the 50<sup>th</sup>, 60<sup>th</sup>, 70<sup>th</sup> etc., or the percentiles of each single subject, were plotted against the drug concentrations in blood corresponding to the same percentiles. The regression curve equations were determined using the "trendline" function in Microsoft Excel. Linear, quadratic and power functions were calculated. Linear functions were preferred if matching the data well. In some cases the highest or lowest percentiles were excluded to obtain better fitting regression curves. For THC, the best fitting prevalence regression formula was obtained when including samples that were positive in only one specimen. More information on prevalence regression has been published previously (61). Prevalence regression was used only for substances where more than 10 paired samples were positive (above the analytical cut-off) for both blood and oral fluid.

#### 10.3.2.4 Comparison of estimation procedures

Three formulae for the estimation of cut-off concentrations in oral fluid were generated based on the use of average OF/B ratio, median OF/B ratio and percentile regression. To determine which formulae fitted the original paired data best, the prevalence of samples above selected cut-off concentrations in blood was estimated using the formulae and compared with the actual prevalence in blood. The accuracies of the three procedures were calculated for the chosen cut-off concentrations in blood and for concentrations corresponding to 2.5 times and 5 times the analytical cut-off. For cocaine, diazepam, methamphetamine and nordiazepam slightly higher concentrations than the cut-off for blood were used for the calculations at the lowest concentration because one laboratory had used a higher cut-off for those analyses. The procedure with the least average percent deviation (in absolute value) from the actual number of subjects with drug concentrations above the cut-offs in blood was identified as the best one for each substance separately.

For substances with OF/B ratios from less than 10 individuals, the cut-off concentration in oral fluid was estimated by multiplying the cut-off concentration in blood with the average OF/B ratio, except for 6-AM where the median OF/B gave a better fit.

### 10.3.3 Results and Discussion

Less than 10 OF/B ratios were available for the following substances: 6-AM, flunitrazepam, MDA, MDEA, MDMA, zopiclone, 7-amino-clonazepam and 7-amino-flunitrazepam. Regression formulae for the remaining substances are presented in Table 12. Prevalence regression curves for benzoylecgonine and THC are presented in Figure 12.

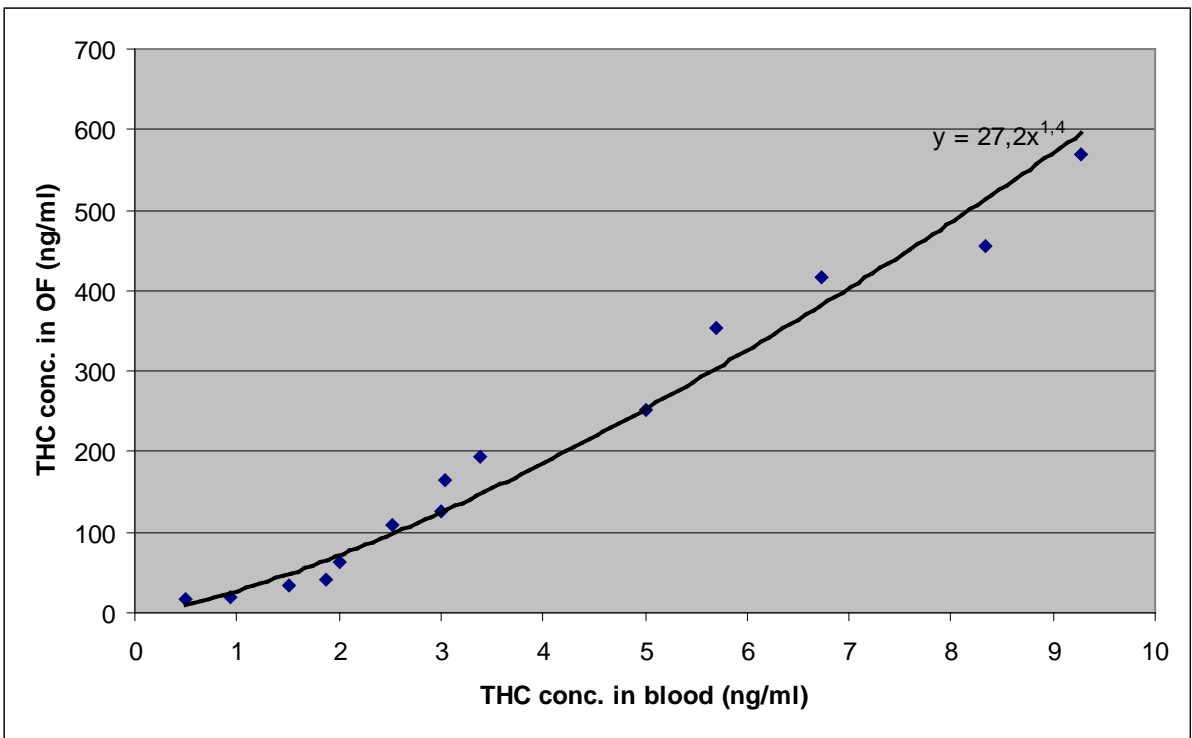
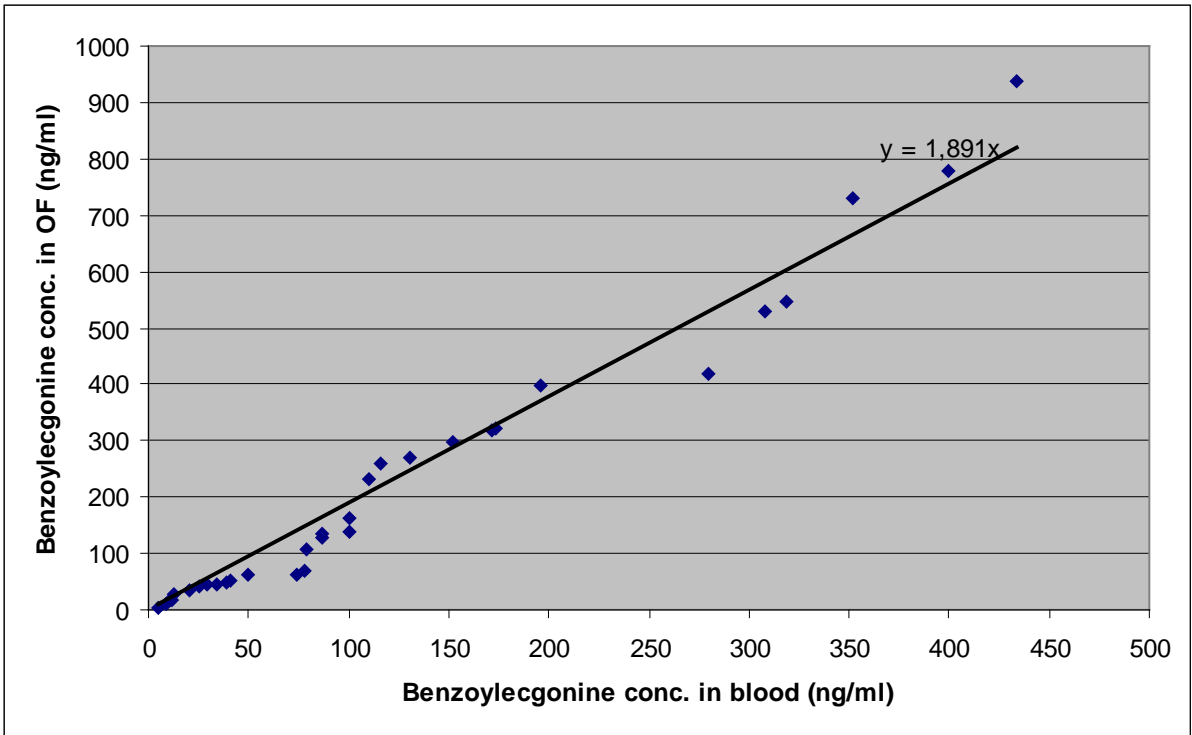


Figure 12: Prevalence regression curves for benzoylecgonine and THC.

A comparison of the accuracies when using percentile regression, average and median OF/B values for estimation of equivalent cut-off concentrations for some drugs are presented in Table 13. Equivalent cut-off concentrations in oral fluid were determined using the methods, which fitted the paired blood and oral fluid concentrations best, see Table 14.

The estimated equivalent cutoff concentrations for oral fluid and blood were used for the calculations of drug prevalence (Deliverable 2.2.3) and for the odds ratio calculations (Deliverable 2.3.5). The DRUID project had originally chosen analytical cut-off concentrations in blood for diazepam and zolpi-

dem of 20 ng/ml. The analytical methods employed for oral fluid had LOQs of 5 and 10 ng/ml, respectively, in some of the participating laboratories. Initial calculations indicated that concentrations in oral fluid that were equivalent to the chosen cut-off concentrations for blood were lower than the LOQs. Therefore, the cut-off concentrations in blood for diazepam and zolpidem had to be increased to 140 and 37 ng/ml, respectively, in order to obtain equivalent cut-off concentrations in oral fluid that were higher than the LOQs for the analytical methods used.

Table 12: Percentile regression formulae for different substances

DRUID core substance	Percentile regression	Slope or formula
Alprazolam	Linear	0.35
Amphetamine	Linear	22.84
Benzoylcegonine	Linear	1.891
Clonazepam	Linear	0.1744
Cocaine	Linear	17.69
Codeine	Linear	$y=6.5x + 29$
Diazepam	Linear	0.0392
Lorazepam	Linear	0.0824
Methadone	Linear	2.164
Methamphetamine	Linear	24.87
Morphine	Linear	7.732
Nordiazepam	Linear	0.0624
Oxazepam	Linear	0.2644
THC	Power	$y=27.2x^{1.39}$
Zolpidem	Linear	0.3067
Tramadol	Quadratic	$y = 0.0205x^2 + 8.5594x$

Table 13: Accuracies for cut-off concentrations in oral fluid that were equivalent to those use for blood for the three estimation methods. Numbers of cases and deviation (%) from 100% accuracy.

Substance (No. total & OF/B)	Actual no.	PR	AV	ME	Best ac- curacy
Alprazolam (n=91 & 64)					
>10 ng/ml	39	31 (-21%)	31 (-21%)	31 (-21%)	AV
>25 ng/ml	23	22 (-4%)	22 (-4%)	22 (-4%)	
>50 ng/ml	14	15 (+7%)	15 (+7%)	15 (+7%)	
Amphetamine (n=73 & 42)					
≥20 ng/ml	43	37 (-14%)	37 (-14%)	39 (-9%)	ME
≥50 ng/ml	31	27 (-13%)	27 (-13%)	29 (-6%)	
≥100 ng/ml	23	19 (-17%)	19 (-17%)	23 (+0%)	
Benzoylcegonine (n=84 & 38)					
≥50 ng/ml	40	37 (-8%)	32 (-20%)	37 (-8%)	PR
≥125 ng/ml	28	28 (+0%)	21 (-25%)	29 (+4%)	
≥250 ng/ml	17	19 (+12%)	17 (+0%)	21 (+24%)	
Clonazepam (n=52 & 33)					
≥10 ng/ml	32	26 (-19%)	25 (-22%)	29 (-9%)	PR
≥25 ng/ml	16	16 (+0%)	15 (-6%)	19 (+19%)	
≥50 ng/ml	6	7 (+17%)	7 (+17%)	9 (+50%)	
Codeine (n=93 & 40)					
≥10 ng/ml	29	29 (+0%)	36 (+24%)	47 (+62%)	PR
≥25 ng/ml	17	16 (-6%)	20 (+18%)	26 (+53%)	

Substance (No. total & OF/B)	Actual no.	PR	AV	ME	Best ac- curacy
≥50 ng/ml	9	10 (+11%)	11 (+22%)	15 (+67%)	
Diazepam (n=81 & 39)					
≥26 ng/ml	52	48 (-8%)	47 (-10%)	50 (-4%)	ME
≥50 ng/ml	47	44 (-6%)	42 (-11%)	47 (+0%)	
≥100 ng/ml	35	36 (+3%)	34 (-3%)	39 (+11%)	
Morphine (n=75 & 34)					
≥10 ng/ml	33	32 (-3%)	30 (-9%)	35 (+6%)	AV
≥25 ng/ml	14	19 (+36%)	17 (+21%)	23 (+64%)	
≥50 ng/ml	8	12 (+50%)	9 (+13%)	14 (+75%)	
Nordiazepam (n=117 & 69)					
≥26 ng/ml	72	62 (-14%)	63 (-13%)	64 (-11%)	AV
≥50 ng/ml	64	52 (-19%)	55 (-14%)	57 (-11%)	
≥100 ng/ml	44	41 (-7%)	44 (+0%)	47 (+7%)	
Oxazepam (n=53 & 20)					
≥50 ng/ml	16	15 (-6%)	20 (+25%)	22 (+38%)	PR
≥150 ng/ml	9	10 (+11%)	12 (+33%)	13 (+44%)	
≥250 ng/ml	8	4 (-50%)	4 (-50%)	11 (+38%)	
THC (n=162 & 55)					
≥1 ng/ml	62	57 (-8%)	58 (-6%)	75 (+21%)	PR
≥2.5 ng/ml	35	34 (-3%)	40 (+14%)	51 (+46%)	
≥5 ng/ml	18	16 (-11%)	29 (+61%)	40 (+122%)	

PR = percentile regression, AV = multiplication with average OF/B, ME = multiplication with median OF/B.

Table 14: Recommended cut-offs for DRUID core substances

Substance	Analytical cut-off concentrations (ng/mL)		Equivalent cut-off concentrations for drug prevalence studies (ng/mL)	
	Blood	Oral fluid	Blood	Oral fluid
6-AM	10	5	10	161
Alprazolam	10	1	10	3.5
Amphetamine	20	25	20	360
Benzoylcegonine	50	10	50	95
Clonazepam	10	1	10	1.7
Cocaine	10	10	10	170
Codeine	10	20	10	94
Diazepam	20	5	140	5.0 <sup>2</sup>
Flunitrazepam	2	1	5.3 <sup>1</sup>	1.0 <sup>2</sup>
Lorazepam	10	1	10	1.1
MDA	20	25	20	220 <sup>1</sup>
MDEA	20	25	20	270 <sup>3</sup>
MDMA	20	25	20	270 <sup>1</sup>
Methadone	10	20	10	22
Methamphetamine	20	25	20	410
Morphine	10	20	10	95
Nordiazepam	20	1	20	1.1

Oxazepam	50	5	50	13
THC	1	1	1.0	27
Zolpidem	20	10	37	10 <sup>2</sup>
Zopiclone	10	10	10	25 <sup>1</sup>
Tramadol	50	50	50	480
7-amino-clonazepam	10	1	10	3.1 <sup>1</sup>
7-amino-flunitrazepam	2	1	8.5 <sup>1</sup>	1.0 <sup>2</sup>

<sup>1</sup> data based on less than 10 individual cases

<sup>2</sup> recommended cut-off lower than the original DRUID cut-off in oral fluid

<sup>3</sup> no positive cases; cut-off of MDMA used for MDEA

It is expected that the studied cohort constitute a representative selection of drivers from Europe, and the results can therefore be used to compare drug prevalence in the countries that participated in the DRUID roadside surveys. However, the estimated equivalent cut-off concentrations were based on a limited number of cases. Therefore, it is expected that similar studies of larger cohorts might give slightly different results.

The equivalent cut-off concentrations are only valid for studies using StatSure Saliva Sampler. The cut-off concentrations for different substances are not equivalent regarding impairment.

## 10.4 The blood spot approach

### 10.4.1 Introduction

The use of dried blood spots (DBS) has already been described by Ivar Bang in 1913 for the determination of the blood glucose concentration in an animal study (62). However, it was not until the early sixties that Robert Guthrie published a DBS method for a neonatal screening to diagnose phenylketonuria by determination of phenylalanine causing a more wide-spread use of this technology (63). This method was the basis for further DBS methods in newborn screening on congenital metabolic disorders (64-66), which are routinely performed today for over two decades. Despite of the limited sample size of 10-100 µL blood, analysis of DBS specimens has become feasible with the advent of increasingly sensitive MS technologies (67). Thus, DBS have recently established themselves as a valuable tool in therapeutic drug monitoring (68). As yet, the use of DBS in samples derived from driving under the influence of drug (DIUD) cases has not been considered at all; and the DRUID project offered a unique opportunity in this respect.

Compared to whole blood and plasma samples, DBS provide many advantages concerning blood sampling, transport, storage, stability and risk of infections. Whereas whole blood and plasma sampling has to be done only by medical personnel, DBS can be prepared using capillary blood after a finger or heel prick. This simple sampling can also be performed by non-medical personnel and is a less invasive alternative to taking of a blood sample. In addition, it is also possible in subjects with limited venous access, such as e.g. injecting drug users. Transport and storage of DBS samples can be performed in sealed envelopes with desiccant packs. Since no additional cooling is necessary, DBS can be sent via regular mail. Because of the absence of water, DBS make labile compounds such as ester type drugs less susceptible to degradation (69). Moreover, the use of DBS decreases the risk of infections with blood-borne viruses. It has been shown that HI-viruses are inactivated in blood samples on filter paper after the spots have been dried at room temperature (70).

In literature, DBS methods using different sample volumes or DBS punch sizes for the determination of analytes have been published, first of all those that are of importance in a clinical setting (67). In addition to amino acids and acylcarnitines, proteins, triglycerides, steroids, bacteria, viruses and antibodies can be determined (65). Sensitive LC-MS/MS techniques also allow the analyses of smaller molecules like non-protein drug substances (67). Besides LC-MS/MS, radioimmunoassay or GC-MS were used for DBS analysis (69, 71).

With respect to all these advantages, DBS have been considered to be a suitable sample material for drug analysis in DUID cases. Since blood sampling can be performed by non-medical personnel it is not necessary to wait for the doctor. Roadside sampling is considered to be most advantageous reflecting the actual blood concentration and hence impairment at the time of the police stop or at the scene. This will obviate the need for a re-estimation of the analytical result back to this point of time which is regularly imprecise if possible at all. Therefore, the major objective of the investigation was to test whether DBS results are comparable to the concentrations determined from whole blood specimens being a major prerequisite for the basic application of DBS analysis. The following paragraph presents the results of DBS analysis of d-amphetamine (n=29), 3,4-methylenedioxy-methamphetamine (MDMA, n=75, 38 of placebo condition, 37 of active treatment), morphine (n=7), hydromorphone (n=16), fentanyl (n=13), oxycodone (n=12), risperidone (n=15), alprazolam (n=33) and zopiclone (n=90, 45 of each placebo or active treatment condition) in authentic samples and their comparison with the results of the determination from corresponding whole blood samples. In case of MDMA, fentanyl, oxycodone, risperidone and alprazolam, determination of major or active metabolites was included.

#### 10.4.2 Materials and methods

A customised collection device for use in the sampling study has been prepared by GE Healthcare (Figure 1, GE Healthcare, Dassel, Germany) from #903 Whatman specimen collection paper which is manufactured from 100% pure cotton linters with no wet-strength additives.

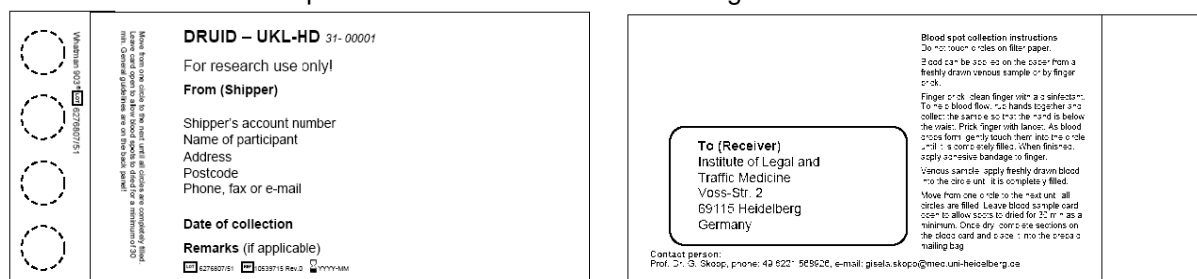


Figure 13: Blood spot card, face and back of the card

This paper is a FDA class II medical device complying with FDA regulations during manufacture. In addition, the post-printing quality of the cards had been checked testing a random sampling of forms from this particular batch for blood absorption time, circle size and caliper. Four preprinted circles were available with dotted line circles on one side of the paper (16 mm internal diameter). Information that should be provided at the face of card was: shipper's account number, name of the participant (anonymous), address, postcode, phone and email. On the back of the card, a detailed instruction is given how the sample should be applied. In addition, all co-operating partners were instructed on the proper method for collecting DBS samples on at least 2 occasions. DBS were dried folding the card along the line where the 2 papers are stacked together at ambient temperature for 3 hours; then, they were stored in a zip lock bag containing a desiccant pad until shipped by regular mail as a diagnostic specimen. Matching whole blood samples were stored at -20°C until shipped on dry ice.

#### 10.4.3 Extraction and quantitative determination

DBS had been prepared by spotting a 100 µL aliquot of whole blood onto the custom made DRUID card. For some of the analytes, analytical assays have been developed while for some others, hitherto existing assays have been modified and downscaled to a sample volume of 100 µL. From both, DBS and 100 µL of whole blood the following analytes have been determined: MDMA, 3,4-methylenedioxyamphetamine (MDA), d-amphetamine morphine, hydromorphone, fentanyl, norfentanyl, oxycodone, noroxycodone, risperidone, 9-hydroxy-risperidone (9-OH-risperidone), alprazolam, hydroxylalprazolam and zopiclone. Before extraction, DBS were completely cut out and transferred into plastic tubes. Analysis was performed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) following liquid/liquid extraction. Only hydromorphone was isolated by solid phase extraction. Each sample was extracted twice; deuterated internal standards were used as far as available.

Table 15 gives an overview on the extraction procedures and mass spectrometry conditions developed for the different analytes.

Calibration lines were prepared separately for either whole blood or blood spots and assessed for linearity using by least squares regression using the ratio of the target analyte peak area to the corresponding internal standard peak area.

#### 10.4.4 Validation

Imprecision, extraction efficiency and bench top stability (24 h) were investigated according to the FDA Guidance for Industry (72). Carryover was checked as described by Bansal and DeStefano (73). Ion suppression or enhancement was determined according to Matuszewski et al. (74). The lower limit of detection (LLOD) and quantitation (LLOQ) was estimated from the calibration curves according to DIN 32465 at a probability of 95% (75).

Data analysis was done using Microsoft Excel®. Agreement of the whole blood and DBS concentrations was further assessed by a Bland-Altman plot (76), which is a method commonly used in clinical chemistry to compare two measurement techniques. The differences between the concentrations of matching pairs are plotted against the averages of the two methods. Horizontal lines are drawn at the mean difference, and at the limits of agreement, which are defined as the mean difference plus and minus the 1.96-fold standard deviation SD of the differences. Agreement between two assays exists if 95% of the values lie within these limits.

#### 10.4.5 Materials and instrumentation

Zopiclone was purchased from Rhône Poulenc Rorer (Cologne, Germany). Risperidone, 9-OH-risperidone and didehydromethylrisperidone (internal standard) were supplied by Janssen-Cilag (Neuss, Germany). Amphetamine, MDMA, MDA, morphine, hydromorphone, fentanyl, norfentanyl, oxycodone, noroxycodone, alprazolam and hydroxyalprazolam as well as their deuterated standards and lorazepam-d<sub>4</sub> were obtained from LGC, Wesel, Germany. High-pressure liquid chromatography (HPLC)-grade acetonitrile and methanol as well as ethyl acetate (≥ 99.5%), toluene (≥ 99.5%), isopropanol (≥ 99.5%), solid NaOH (≥ 99%), ammonium acetate (≥ 98%), acetic acid (100%) were from Roth (Karlsruhe, Germany). Isoamyl alcohol (≥ 99%), dichloromethane (≥ 99.8%), ammonium hydroxide (25%), hydrochloric acid (25%), sodium carbonate (≥ 99.5%), sodium hydrogen carbonate (≥ 99.5%), potassium chloride (≥ 99.5%) and boric acid (≥ 99.8%) were supplied by Merck (Darmstadt, Germany). Double distilled water was obtained from Braun (Melsungen, Germany). Drug-free whole blood for preparation of calibration lines and validation standards were purchased from the local blood bank of the University Hospital of Heidelberg. For solid phase extraction of hydromorphone, Bond-Elut C8 1 mL columns were used (Varian, Darmstadt, Germany).

LC-MS/MS analysis was performed on an API 4000 tandem MS with a Turbolon ionization source operated in the positive-ion mode (AB Sciex, Darmstadt, Germany). It was interfaced to an HPLC pump equipped with an autosampler 1100 series, Agilent, Waldbronn, Germany).

Table 15: Analysis of d-amphetamine, MDMA, MDA, morphine, hydromorphone, fentanyl, norfentanyl, oxycodone, noroxycodone, risperidone, 9-OH-risperidone, alprazolam and zopiclone: extraction, chromatography and mass spectrometry conditions

analyte	adjustment of the pH-value for extraction	internal standard (IS)	extracting agent [vol%]	mobile phase A:B:C <sup>A</sup> [v:v:v]	flow [µL/min]	retention time [min]	column	transition used for quantitation	transition IS
MDMA <sup>1</sup>	0.01 M NaOH	MDMA-d <sub>5</sub>	ethyl acetate	60:8:32	220	2,15	♦	194→163	199→165
MDA <sup>1</sup>		MDA-d <sub>5</sub>				2,08		180→163	180→135
d-amphetamine <sup>1</sup>		amphetamine-d <sub>5</sub>				1,71		136→91	141→124



analyte	adjustment of the pH-value for extraction	internal standard (IS)	extracting agent [vol%]	mobile phase A:B:C <sup>Δ</sup> [v:v]	flow [μL/min]	retention time [min]	column	transition used for quantitation	transition IS
morphine <sup>2</sup>	borate buffer pH 8.5	morphine-d <sub>3</sub>	ethyl acetate	50:10:40	220	2.00	♦	286→152	289→152
hydromorphone <sup>3</sup>	carbonate buffer pH 9.0	hydromorphone-d <sub>3</sub>	dichloromethane/ isopropanol/ conc. NH <sub>3</sub> 80:20:2	50:10:40	300	1.18	#	286→185	289→185
fentanyl norfentanyl	5%NH <sub>3</sub>	fentanyl-d <sub>5</sub> norfentanyl-d <sub>5</sub>	ethyl acetate	40:12:48	250	1.69 1.36	#	337→188 233→84	342→188 238→84
oxycodone noroxycodone	5%NH <sub>3</sub>	oxycodone-d <sub>6</sub> noroxycodone-d <sub>6</sub>	ethyl acetate	50:10:40	300	1.20 1.11	#	316→298 302→284	322→304 305→287
risperidone 9-OH- risperidone	borate buffer pH 8.5	didehydromethyl- risperidone	ethyl acetate	50:10:40	300	2.00 1.90	#	411→191 427→207	421→201
alprazolam hydroxyal- prazolam	borate buffer pH 8.5	alprazolam-d <sub>5</sub>	tolu- ene/isoamyl alcohol 95:5	45:11:44	250	3.70 2.99	#	309→205 325→297	314→210 330→302
zopiclone	borate buffer pH 8.5	lorazepam-d <sub>4</sub>	tolu- ene/isoamyl alcohol 95:5	40:12:48	300	1.15	#	389→245	325→307

<sup>Δ</sup> A: 4 mM ammonium acetate buffer pH 3,2; B: methanol; C: acetonitrile

# Phenomenex Luna C<sub>18</sub> 2,0 mm x 150 mm, particle size 5 μm, Phenomenex, Aschaffenburg, Germany

♦ Agilent Zorbax Eclipse XDB-C<sub>8</sub> 2,1 mm x 150 mm, particle size 5 μm, Agilent, Waldbronn, Germany

<sup>1</sup> the organic phase was acidified with 50 μL of methanol HCl (49:1, v:v) prior to evaporation to dryness

<sup>2</sup> ultrasonication (5 min) was applied following addition of borate buffer and IS

<sup>3</sup> solid phase extraction

## 10.4.6 Results and discussion

### 10.4.6.1 Evaluation

Table 16 gives an overview on the validation results determined in DBS. Additionally, matrix effect, extraction efficiency and 24 h bench top stability were checked; all values were within acceptable ranges (data not shown). It could also be observed that matrix effects in DBS are of a lesser extent than in whole blood (data not shown). Matrix effects exerting a serious impact on the results are generally considered as a major drawback of LC-MS/MS analysis. Carryover could not be observed for any analyte. There were no significant differences between the validation results in blood and DBS; all parameters were in the same range as presented in Table 16 or better.

Table 16: Validation results for the most important substances determined in plasma

analyte	LLOD [ng/mL]	LLOQ [ng/mL]	between-run precision [%]	within-run precision [%]	linearity
<b>amphetamine</b>	0.7	2.6	12 ng/mL: 3.9 40 ng/mL: 5.2	12 ng/mL: 2.6 40 ng/mL: 3.3	5-40 ng/mL r=0.9999
<b>MDMA</b>	0.4	1.4	20 ng/mL: 4.9 250 ng/mL: 7.8	20 ng/mL: 3.6 250 ng/mL: 3.6	A: 50-400 ng/mL r=1.0000 B: 5-40 ng/mL r=0.9997
<b>MDA</b>	0.1	0.5	5 ng/mL: 4.6 15 ng/mL: 6.6	5 ng/mL: 3.8 15 ng/mL: 5.0	A: 5-40 ng/mL r=0.9999 B: 0.5-4 ng/mL r=0.9999
<b>morphine</b>	1.3	4.8	250 ng/mL: 5.6	250 ng/mL: 5.6	50-500 ng/mL r=1.0000
<b>hydromorphone</b>	0.4	1.4	4 ng/mL: 3.4 12 ng/mL: 3.6	4 ng/mL: 1.8 12 ng/mL: 1.4	2-20 ng/mL r=1.0000
<b>fentanyl</b>	0.02	0.08	0.25 ng/mL: 4.0 6.5 ng/mL: 4.1	0.25 ng/mL: 3.3 6.5 ng/mL: 2.4	0.1-10 ng/mL r=1.0000
<b>norfentanyl</b>	0.02	0.08	0.25 ng/mL: 4.9 2.0 ng/mL: 3.8	0.25 ng/mL: 1.1 2.0 ng/mL: 2.0	0.1-4.0 ng/mL r=1.0000
<b>oxycodone</b>	0.1	0.3	10 ng/mL: 6.7 100 ng/mL: 3.6	10 ng/mL: 2.7 100 ng/mL: 3.6	5-100 ng/mL r=1.0000
<b>noroxycodone</b>	0.1	0.4	10 ng/mL: 5.7 80 ng/mL: 3.1	10 ng/mL: 5.2 80 ng/mL: 2.5	5-100 ng/mL r=0.9989
<b>risperidone</b>	0.3	1.2	6.7 ng/mL: 3.1 19.75 ng/mL: 3.9	6.7 ng/mL: 2.6 19.75 ng/mL: 3.9	5-25 ng/mL r=0.9997
<b>9-OH-risperidone</b>	0.3	1.3	10 ng/mL: 3.7 40 ng/mL: 5.8	10 ng/mL: 2.1 40 ng/mL: 4.9	5-60 ng/mL r=0.9999
<b>alprazolam</b>	0.2	0.7	5 ng/mL: 7.2 30 ng/mL: 5.2	5 ng/mL: 5.3 30 ng/mL: 4.0	2.5-50 ng/mL r=0.9999
<b>zopiclone</b>	0.1	0.3	10 ng/mL: 6.0 50 ng/mL: 4.2	10 ng/mL: 2.5 50 ng/mL: 2.0	10-50 ng/mL r=0.9999

10.4.6.2

### 10.4.6.3 Concentrations of analytes determined from DBS and comparison of the results with those of whole blood

All results > LLOQ of DBS analysis are summarised in Table 17.

Table 17: Results of DBS analysis obtained from authentic samples

analyte	n>LLOQ	concentration range [ng/mL]	mean [ng/mL]	median [ng/mL]
d-amphetamine	29	10.9-43.9	21.6	20.4
MDMA	36	2.5-447.5	181.3	172.7
MDA	32	0.9-24.1	8.9	8.6
morphine	7	82.2-472.6	238.1	209.2
hydromorphone	15	2.4-19.3	8.7	7.6
fentanyl	13	0.18-6.27	1.26	0.49
norfentanyl	13	0.07-2.28	0.47	0.21
oxycodone	12	19.3-123.6	61.2	57.1
noroxycodone	12	20.4-86.5	42.6	33.8
risperidone	11	1.2-20.8	9.5	6.2
9-OH-risperidone	14	4.2-30.8	14.7	14.7
alprazolam	28	2.5-20.1	6.2	5.2
zopiclone	45	9.7-39.8	22.1	21.5

With respect to the advantages mentioned in the introduction, DBS may be a suitable method for roadside blood sampling in case of suspected DUID. Before DBS can be used for drug testing, it must be shown that DBS analysis is able to provide results that are as reliable as those using whole blood samples. Therefore, blood and matching DBS specimens were provided for all drugs investigated during the particular DRUID studies in Germany, Italy, Greece and The Netherlands. Results of both media were compared using the DBS/blood ratio (DBS/b) and Bland-Altman analysis. Accordingly, the respective mean of the corresponding results determined with the two different methods were plotted on the x-axis and their difference on the y-axis. In contrast to a scatter plot of blood and corresponding DBS concentrations, the Bland-Altman difference plot allows to assess the distribution of the differences over the whole concentration range. The mean of the differences indicates an over- or underestimation by one of the two methods. Besides the mean of the differences, the 95% limits of agreement were calculated as the mean difference  $\pm 1.96 \cdot SD$ . In case of a normal distribution, 95% of the differences are expected to lie within these limits (76). Ideally, the mean DBS/b ratio should be equal to 1.00, which means that results from whole blood and DBS analysis do not differ.

Table 20 in the appendix provides information on the results of the analysis of the investigated drugs in whole blood. Hydroxyalprazolam could be detected either at a concentration < LLOQ or not at all due to the short time between drug administration and collection of the blood specimens. Therefore, this analyte has not been considered further. All analytes and their corresponding DBS/b ratios including respective RSD are summarised in Table 18.

Table 18: Summary of DBS/b ratios and RSDs

analyte	n	DBS/b range	DBS/b mean	RSD [%]
d-amphetamine	29	0.94-1.15	1.05	5.23
MDMA	36	0.92-1.09	1.01	2.89

analyte	n	DBS/b range	DBS/b mean	RSD [%]
MDA	32	0.85-1.26	1.03	6.44
morphine	7	0.95-1.02	0.99	2.22
hydromorphone	15	0.86-1.08	0.99	6.36
fentanyl	13	0.90-1.09	1.00	8.56
norfentanyl	13	0.84-1.05	0.97	5.94
oxycodone	12	0.98-1.10	1.02	3.52
noroxycodone	12	0.89-1.06	1.00	4.32
risperidone	10	0.86-0.97	0.93	3.51
9-OH-risperidone	14	0.91-1.03	0.97	4.56
alprazolam	28	0.92-1.25	1.03	6.79
zopiclone	45	0.63-1.22	0.86	15.86

For all samples containing opioid type drugs the estimated DBS/b ratios were in an acceptable range and showed a small range of variation. Also, ratios and RSDs for the benzodiazepine-type drug alprazolam as well as for amphetamine derivatives such as MDMA, MDA and d-amphetamine indicate that methods for blood and DBS determination do not differ.

As an example, MDMA could be quantified in DBS as reliably as in whole blood specimens which was already evident from the DRUID deliverable of July 2010 (77). The results obtained in the current study confirmed once again equality of both methods. In addition, equivalence of the methods could be proven for MDA, which is a minor but active metabolite of MDMA: The DBS/b ratio of 1.03 is very close to 1.00; a very low coefficient of variation of 6.44% and a very small mean difference between both methods could be estimated from the results. Figure 14 shows the Bland-Altman difference plot obtained for MDA in DBS and whole blood:

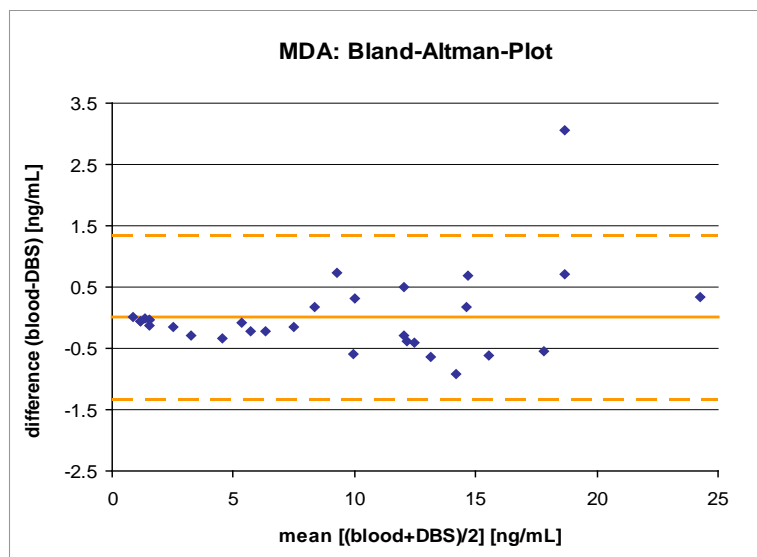


Figure 14: Bland-Altman difference plot for MDA. The solid line illustrates the mean difference of 0.02 ng/mL, the dotted lines indicate the limits of agreement set to  $1.96 \cdot SD$  (-1.36 and 1.40 ng/mL).

All values except for a single one are within the limits of agreement. No trend of the differences between the results obtained from either blood or DBS values over the whole concentration range could be observed. Especially, the very small mean difference leaves no doubt that analysis of MDA from DBS is as reliable as from whole blood. This conclusion could also be drawn from the Bland-Altman analysis of all other analytes under investigation except zopiclone. No trend of the differences over the concentration range could be observed for any of the substances under investigation. Additionally, the number of outliers was always below 5%. Also, DBS/b ratios were very close to 1.00.

Contrary, the DBS/b ratio of zopiclone being 0.86 indicates an overestimation of the results from blood compared to DBS. This overestimation is supported by the mean difference calculated for the Bland-Altman difference plot of zopiclone (Figure 15).

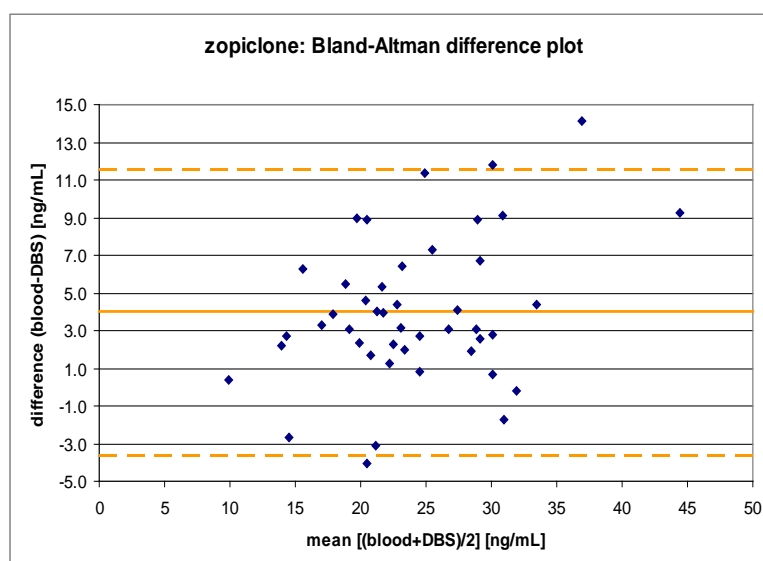


Figure 15: Bland-Altman difference plot for zopiclone. The solid line illustrates the mean difference of 3.99 ng/mL, the dotted lines indicate the limits of agreement set to  $1.96 \cdot SD$  (-3.62 and 11.59 ng/mL).

Whole blood samples were stored at  $-20^{\circ}\text{C}$  until analysis, whereas DBS were kept at ambient temperature. With respect to the different temperatures of storage, degradation of zopiclone to 2-amino-5-chloropyridine might have occurred which has recently been published for whole blood samples by Nilsson et al. (78). Currently, a stability investigation is in process to compare the degradation in whole blood and DBS at the same storage conditions. Results may enable a conclusion concerning the best storage conditions and whether determination from DBS is superior to that of whole blood.

In addition to the DBS/b ratios presented in Table 18, Table 19 gives the results of the Bland-Altman analyses for all analytes. Concentrations determined from corresponding whole blood samples are summarised in Table 20.

Table 19: Summary of results of the Bland-Altman difference plots.

#: the lowest DBS concentration of risperidone was not considered for Bland-Altman analysis, due to the fact that the corresponding blood concentration was below the LLOQ of whole blood analysis.

analyte	mean difference blood-DBS [ng/mL]	mean-1.96xSD [ng/mL]	mean +1.96xSD [ng/mL]	mean difference/mean blood concentration [%]
d-amphetamine	-1.03	-3.32	1.25	-5.01
MDMA	3.55	-14.34	7.25	-1.94
MDA	0.01	-1.33	1.35	0.09
morphine	2.12	-8.30	12.53	0.88
hydromorphone	0.14	-0.90	1.17	1.53
fentanyl	-0.02	-0.19	0.15	-1.76
norfentanyl	-0.006	-0.068	0.055	-1.34
oxycodone	-1.24	-4.46	1.98	-2.07
noroxycodone	0.27	-3.26	3.80	0.63
risperidone#	0.83	-0.67	2.32	7.44

<b>9-OH-risperidone</b>	0.64	-1.13	2.40	4.15
<b>alprazolam</b>	-0.11	-1.01	0.80	-1.81
<b>zopiclone</b>	3.99	-3.62	11.59	15.30

Obviously, for no analyte except zopiclone the mean difference exceeded  $\pm 10\%$  of the mean blood concentration. Therefore, DBS and whole blood methods for the analytes investigated during the DRUID project can be regarded to be equivalent.

#### 10.4.6.4 Conclusions

The DBS assay has potential as a precise and inexpensive option for the determination of several analytes in small blood samples. The small sample volume of 100  $\mu\text{L}$  requires very sensitive techniques. By using LC-MS/MS, all analytes investigated in the presented study could be determined with sufficient LLOQs. Evaluation data showed no significant differences in precision as well as LLODs and LLOQs. Analysis of DBS is feasible with the advent of increasingly sensitive MS technologies such as LC-MS/MS. Although optimization of extraction procedures is necessary, DBS analysis turned out to be superior to determination from whole blood with regard to matrix effects.

The DBS/b ratios were very close to 1.00, and the relative standard deviations  $\leq 8.56\%$ . Measures of under-/overestimation were readily provided by Bland-Altman difference plots, and 95% of all differences between the concentrations determined from either whole blood or DBS were within the limits of agreement. Also, differences were uniformly distributed across the concentration ranges. Except zopiclone, which is very sensitive to degradation, all substances investigated in the presented studies could be determined in DBS as reliably as in whole blood specimens. A zopiclone stability study in blood and DBS samples is running to compare the extent of degradation in both media: then, conclusions can be drawn concerning the optimum sample material and optimum temperature for storage.

The use of DBS in routine analysis will result in simplified handling during blood sampling, transport and storage as well as sample processing in the laboratory. The present device had well characterised properties with regard to blood volume/unit area and chromatography. Considering that the blood spot size depends on the hematocrit value for analytes whose distribution between blood and plasma differs from one, extraction of the whole DBS is recommended (79).

Based on the present results, DBS drug analysis can be regarded as a valuable and inexpensive alternative to determination from whole blood. We are quite confident that the use of DBS will facilitate blood analysis in DUID cases in the near future.

#### 10.4.6.5 Appendix

Table 20: Summary of the results obtained from whole blood.

analyte	range[ng/mL]	mean [ng/mL]	median [ng/mL]
d-amphetamine	10.8-40.7	20.6	19.6
MDMA	2.8-444.3	177.9	171.0
MDA	0.86-24.4	8.9	9.0
morphine	82.4-479.1	240.2	215.7
hydromorphone	2.3-20.2	8.8	7.0
fentanyl	0.18-6.10	1.24	0.52
norfentanyl	0.08-2.21	0.46	0.21
oxycodone	17.8-122.6	59.9	53.7
noroxycodone	20.6-91.2	42.9	33.6
risperidone	4.3-22.7	11.1	6.6
9-OH-risperidone	4.1-29.8	15.3	15.5
alprazolam	2.1-20.7	6.0	5.2
zopiclone	10.1-49.1	26.1	24.9

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