Thesis for the degree Master of Pharmacy

ANALYSIS OF SELECTED BENZODIAZEPINES IN THE ENVIRONMENT BY HF-LPME AND LC-MS/MS

By

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PREFACE

This master thesis was performed at the University of Tromsø (UIT), Department of Pharmacy. All analytical work was performed during the period October 2008 - May 2009.

Dr. Terje Vasskog at the department of medical chemistry, UIT, has been the chief internal supervisor. Professor Einar Jensen has been the second supervisor

ACKNOWLEDGEMENTS

First of all I would like to express my gratitude to my excellent supervisor
Dr. Terje Vasskog. His support, help and guidance through this master work has been priceless.
I would like to thank Professor Einar Jensen for introducing me to the analytical work and for encouragement through the laboratory work.

Then I would like to thank Jens Rundhaug at Tromsø Water and Drainage, for all help during collection of waste water samples.
PhD student Terkel Hansen for all help and discussions concerning basic and acidic hydrolysis of benzodiazepines.
I would also like to thank my class mate Kristian L. Forsberg for all help regarding the pictures included in this thesis.

Finally I would like to thank my family and especially my parents for their love and support through the years of my education. They have always stood by my side, believed in me and supported me. I would not have made it without you gays.

Tromsø, May 2009

Ingjerd Heiberg Jakobsen

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1 ABSTRACT

Industrial chemicals, pesticides and other similar compounds are all known to be a burden for the environment. In high concentrations they can all affect the biological environment in a manner that is dangerous for organisms. The presence of pharmaceuticals and personal care products (PPCPs) in the environment are of great interest regarding to their potential toxicity. Pharmaceuticals and PPCPs are discovered in soil, sludge, sewage and in the adjacent aquatic environment.

The aim of this study was to develop and optimize a hollow fiber liquid- phase microextraction (HF-LPME) method for extraction and pre-concentration of benzodiazepines and its metabolites in sewage water. Different parameters like donor- and acceptor phases, fibers and organic phases were to be tested. It is preferred to perform extraction without the use of synthetic organic solvents. An organic phase using e.g. plant oils possess lower health risk, lower costs and have no restrictions regarding deposition. Different plant oils, essential oils and volatile oils were therefore tested as organic phase.

The compounds that was to be investigated in this study were chosen by looking at sales statistics for benzodiazepines in Norway over a three years period, 2005-2007[1]. Some metabolites of the compounds were also included. The chosen compounds were zopiclone, zopiclone-d8, zopiclone N-oxide, N-desmethyl zopiclone hydrochloride, zolpidem, zolpidem-d6, alprazolam, alprazolam 5-oxide, 1-hydroxy alprazolam, midazolam, midazolam –d5 and 1`-hydroxy midazolam. Later in the project clonazepam and 7-aminoclonazepam were included.

Sewage water samples were collected at Langnes Sewage Treatment Plant (STP), Tromsø, before they were filtered and extracted by hollow fiber liquid phase microextraction and analysed on Ultra Performance Liquid Chromatography- Mass Spectrometry/ Mass Spectrometry (UPLC- MS/MS).

Quantifiable amounds of zolpidem was found during the collection in January, midazolam and 1- hydroxyl midazolam were detectable. In April 1- hydroxy midazolam, midazolam and zolpidem were detectable.

2 ABBREVIATIONS

DDD	Defined Daily Dosages
DDT	Dichlorodiphenyltrichloroethane
GC	Gas Chromatography
HF- LPME	Hollow Fiber- Liquid Phase Microextraction
L	Liter
LOD	Limit Of Detection
Log- D	Distribution coefficient
LOQ	Limit Of Quantification
M	Molar
m/z	*Mass- to- charge ratio*
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Mass Spectrometry /Mass Spectrometry
POP	Persistent Organic Pollutants
PPCP	Pharmaceuticals and Personal Care Products
RPM	Rounds Per Minute
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
STP	Sewage Treatment Plant
UPLC	Ultra Performance Liquid Chromatography

3 INTRODUCTION

Persistent organic pollutants (POP`s) and other compounds have for many years been investigated regarding their influence on the environment. Most POP`s found are products from industrial production all over the world e.g. Dichlorodiphenyltrichloroethane (DDT) used in insecticides[2].

The general definition of environmental pollutants is substances that have high acute toxicity, tendencies to undergo bioaccumulation and biomagnifications. They also have a high resistance against degradation and long half life in the environment. A longer time of exposure heightens the risk for multiple contamination of the ecosystem[3]. POP`s become widely distributed geographically and can accumulate in the fatty tissue of living organisms. The international community has called for urgent global actions to reduce and eliminate the release of these chemicals to the environment. Another concern is pharmaceuticals found in environmental samples. In 1999, over 50 individual pharmaceuticals and personal care products (PPCP) or their metabolites had been identified in environmental samples[4]. The finding of pharmaceuticals in environmental samples like sludge, soil, sewage water and adjacent seawater has the last years increased the concern of environmental toxicity. Studies regarding environmental toxicity have shown adverse effects on non target organisms in the presence of pharmaceuticals[3]. The list of pharmaceuticals found in environmental samples is increasing, but our knowledge towards the toxic effect they exhibit on wildlife and other non target organisms is still limited.

3.1 Pharmaceuticals in the environment

The last decade the consumption of pharmaceuticals has increased extensively.
In 2008 is was consumed drugs for about 17,6 billions NOK or about 3700 NOK per inhabitant in Norway[5]. There where 1882 pharmaceuticals in the Norwegian market, 1447 different active compounds[6]. Pharmaceuticals have several pathways of entering the environment. Human and veterinary pharmaceuticals are the main source of pharmaceuticals and their metabolites in the environment. The pharmaceuticals reach the environment through mainly excretion of urine or faeces[2]. Before excretion pharmaceuticals are metabolized to a certain extent. They can be structurally modified bye germs in the stomach/ intestine or by enzymatic degradation in the liver. The metabolism may lower activity and enhance water solubility, however in the case of pro-drugs activation occurs. Another way of entering the environment is by chemical dumping. In stead of returning unused pharmaceuticals to the pharmacy, who is responsible for destruction, people use inappropriate disposals as toilets, sinks and garbage. According to the Norwegian Medicines Agency should all applications for marketing license include an assessment regarding possible environmental toxicity. Marketing license of veterinarian pharmaceuticals may be denied if the compound has shown environmental toxicity[7].
Pharmaceuticals are complex molecules, developed and used because of their specific and high biological activity[3]. Because of this specificity and high stability they might have the opportunity to cause harm in even lower concentrations than other POP`s detected in the environment. Most pharmaceuticals do not have an acute toxicity in the concentration ranges found in environmental samples. However, their ability to accumulate and the low dose long-term exposure may affect non target organisms. There have previously been detected pharmaceuticals in waste water, soil, sludge, adjacent seawater and even in drinking water. Contraceptives, anti depressants and antibiotics have all previously shown adverse affects on fish and other non target organisms[3].
The knowledge about how pharmaceuticals affect the environment is limited, especially when it comes to understanding the long-term effects of exposure. Until now the general possible effects of only a few pharmaceuticals have been studied. More studies regarding pharmaceuticals in the environment have to be performed in the future to truly understand their effects.

3.2 Benzodiazepines

According to the Norwegian health institute, 670.000 Norwegians were prescribed a benzodiazepine or benzodiazepine like drug in 2007. Benzodiazepines are by far the most commonly prescribed hypnotics. Drugs that affect the nervous system, like benzodiazepines, represented one fifth of the total amount drugs prescribed in Norway, 2008[8]. They all have sedative/hypnotic, anxiolytic, amnesic, muscular relaxant and anticonvulsant actions with minor differences in the relative potency of these effects[9, 10]. Hypnotics are generally intended for short- time use, they are discontinued as soon as possible. As class benzodiazepines has been associated with abuse and dependence. Hangover effects at higher dosages, including drowsiness, confusion, and lack of coordination and slowed reaction time are all registered adverse effects using benzodiazepines. The benzodiazepines exert their action on specific benzodiazepine receptor sites in the body, gamma- aminobutyric acid A($GABA_A$) receptors[2].These receptors mediates fast inhibitory synaptic transmission throughout the central nervous system. The many side effects observed using benzodiazepines has resulted in the development of a number of alternatives. These newer drugs are structurally different from the benzodiazepines and have a short time of duration, but act at the same or similar receptor sites as the original benzodiazepines. Zopiclone and Zolpidem are examples of such newer drugs. They are chemically unrelated to benzodiazepines but have the same pharmacological effect[2, 9]. Because of fewer side effects, Zopiclone is the most prescribed benzodiazepine in Norway.

The basic chemical structure of benzodiazepines consists of a seven- membered ring fused to an aromatic ring. The aromatic ring has four main substituent groups that can be modified without loss of activity(R), see figure 1[11].

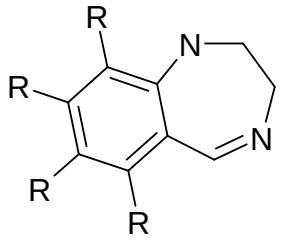


Figure 1: Fundamental chemical structure of benzodiazepine[11].

3.3 Liquid phase microextraction

LPME has been used as extraction technique of analytes since 1996.
The first method developed was two- phase microextraction. This method is based on a single droplet organic solvent hanging from a micro syringe needle. The droplet was placed in an aqueous sample for extraction based on passive diffusion from the aqueous sample into the organic phase. The organic solvent was then withdrawn into the syringe and injected into the GC-MS.

$$\mathbf{A}_{\text{Sample}} \leftrightarrow \mathbf{A}_{\text{Acceptor}}$$

In 1999 a new LPME technique was developed, Hollow Fiber Liquid Phase MicroExtraction (HF-LPME). HF-LPME presents better extraction efficiency and sensitivity compared to two- phase microextraction. The HF-LPME system could consist of two- or three- phases. In a two phase system the analyste in the aqueous sample are extracted into an organic solvent immobilized in the pores and the lumen of the fiber.
The three- phase system consists of an aqueous sample with analytes, which are extracted from the aqueous sample through a thin film of organic solvent in the pores of the hollow fibre, and into an aqueous acceptor phase.
The LPME method is suited for analytes, acidic or basic, with ionizable functional groups. For extraction of basic compounds like benzodiazepines, the pH of the sample has to be adjusted into the alkaline region to promote extraction into the organic phase. To promote high extraction efficiency from the organic phase into the acceptor phase, the pH in the acceptor phase is adjusted to a low acid region [12-15]. In addition the extraction is carried out with stirring at 800 rpm to promote the diffusion.
Liquid phase microextraction is known to perform high pre-concentration and sample clean-up[16]. The basic set-up for three-phase LPME is illustrated in figure 2.

$$\mathbf{A}_{\text{Sample}} \leftrightarrow \mathbf{A}_{\text{Organic acceptor}} \leftrightarrow \mathbf{A}_{\text{Aqueous acceptor}}$$

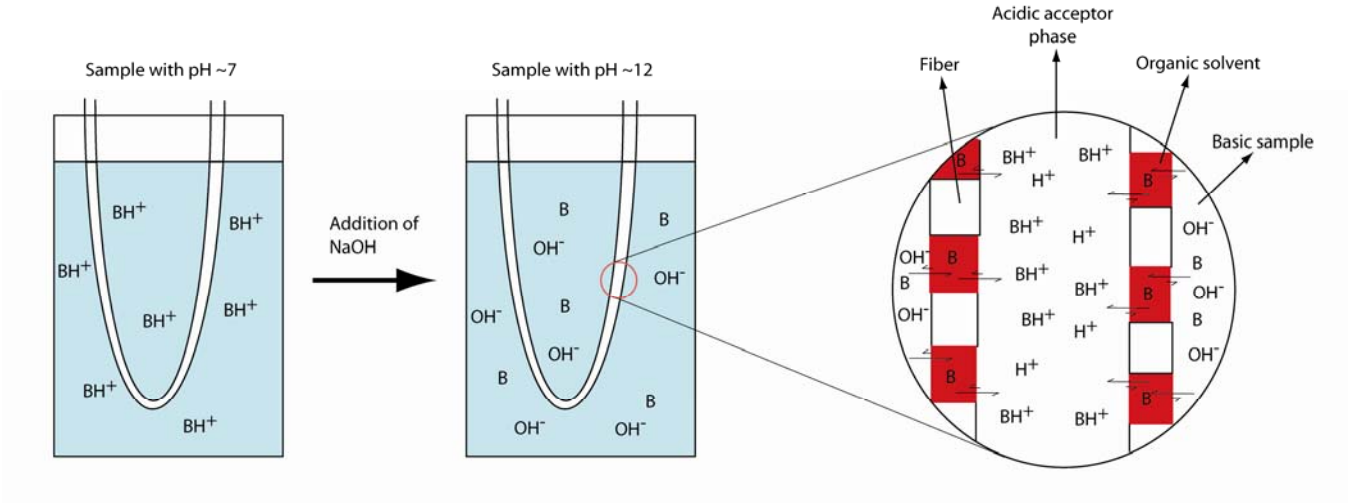


Figure 2: Three-phase Liquid phase microextraction of a basic drug [17].

Selection of organic phase

The organic solvent used to fill the pores of the hollow fiber plays an important role in three-phase LPME, effecting both recoveries and extraction kinetics. Low volatility to prevent evaporation, low polarity to ensure compatibility with the hollow fiber and to prevent leakage into the sample, and low viscosity to ensure rapid mass transfer are important qualities looked for in an organic phase used for this purpose. The organic solvent should also provide high distribution constants for the target analytes[16].

3.4 Ultra performance liquid chromatography

During liquid chromatography a mobile phase is pushed through a column packed with a material for separation of the analytes in the sample. The sample is injected into the mobile phase before the entrance of the column. The columns used are usually 5-25 cm long, their task together with the mobile phase is to separate the analytes in the sample[18]. Typically the stationary phase of an UPLC- column has spherical particles with smaller diameter than the particles used in HPLC- columns. Narrower column and smaller sorbent particles contribute to better sensitivity and lower detection limits.
UPLC is known to give increased resolution and narrower peaks, compared to HPLC. Narrower peaks may result in better sensitivity depending on the selected detector. The sample run may be made more efficiently and therefore making UPLC a more cost-effective method[19].

Reverse phase is the most used separation technique in liquid chromatography. It consists of a hydrophobic stationary phase (column) and a hydrophilic mobile phase. The more hydrophobic the compounds, the more they are adsorbed to the hydrophobic stationary phase. The stronger the analytes are adsorbed to the stationary phase, the longer is the retention time for the compound [18]. C_8 (octyl) or C_{18} (octadecyl) bonded silica-based materials is the most used reverse phase columns.

Selection of mobile phase
Liquids used as a mobile phase should be; non reactive, have low viscosity, a certain purity degree and not be flammable. Low viscosity ensures that the pressure in the HPLC- system is kept at low levels. Water, methanol and acetonitrile are among the most frequently used liquids in reverse phase columns. In order to optimize the ionization of the analytes the pH of the mobile phase is adjusted[18].

3.5 Mass Spectrometry

During electrospray ionization the sample is transformed to an aerosol. This is done by applying high voltage to the capillary. Nitrogen is used as an assistant to improve the nebulization. By the outlet of the stainless steel capillary the aerosol passes a cylindrical electrode. Between the capillary and this electrode there is a potential of e.g. 3- 3,5 kW, which charges the aerosol (see figure 3). When the source is operated in positive- ion mode the analytes receives a positive charge. During the analytes flight between the ESI (electro spray ionisation) steel capillary and the entrance of the MS, neutral solvent molecules evaporate from the surface of the droplet. This results in a decrease of the droplet size and a charge- transfer to the analytes. This gas phase ions is led into the MS by an optimized electric voltage. Electric voltage on the cone is optimized in order to guide the analyte ions into the mass spectrometer without causing unnecessary fragmentation in the ion source.

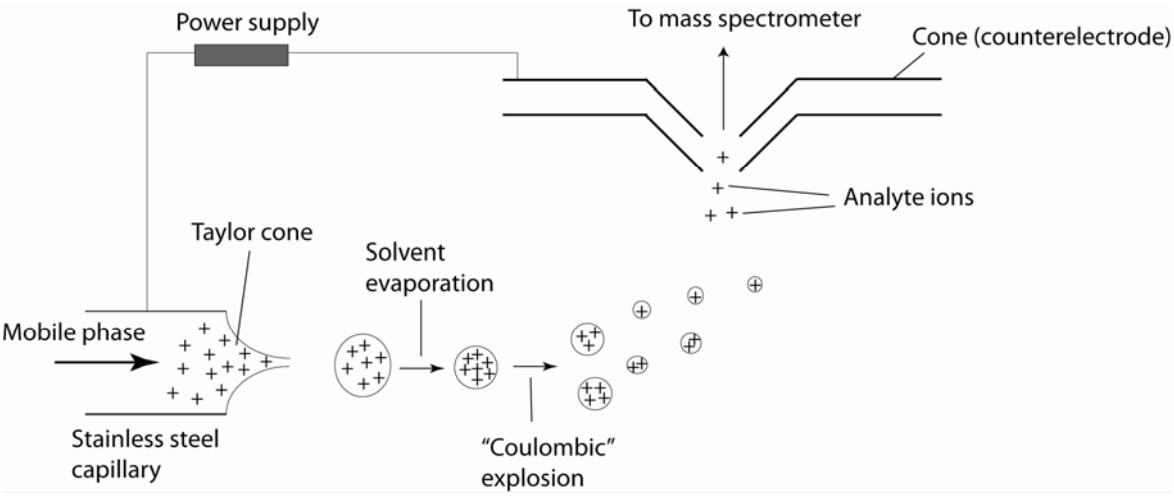


Figure 3: Schematic picture of the Electro spray ionization [17].

MS/MS:

Mass spectrometry is a specific and sensitive method, used for both quantitative and qualitative messurements. During mass spectrometry the analytes are ionized and separated by the mass- to –charge ratio(m/z)[18]. The MS used in this study contain three quadrupoles (Q_1,q and Q_2), in MRM- mode. Q_1 is set to only pass the ions with the right molecular weight. In the collision cell the analytes are fragmented by a non-reactive collision gas (Argon gas). In Q_2 the mass of the fragments are measured, and the most intense product ion is selected for quantitative measurements. See figure 4.

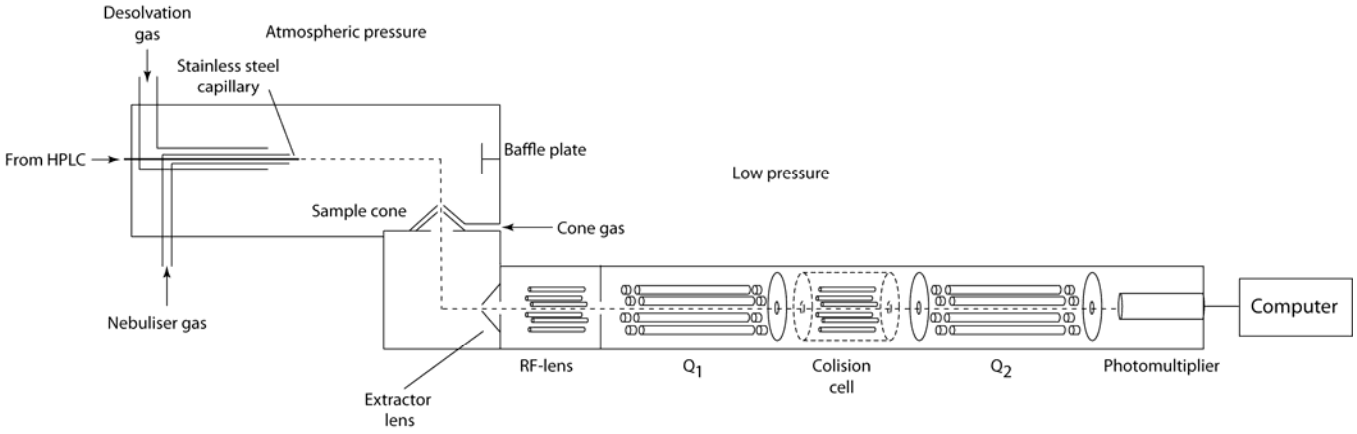


Figure 4: Schematic drawing of the Micromass Quatro- LC mass spectrometer[17].

3.6 Limit Of Detection and Limit Of Quantification

The Limit Of Detection (LOD) is the lowest concentration at which the analyte with certainty can be detected in the sample analyzed with the given method. The analyte could be detected if the concentration is the same or higher than the LOD.
The Limit of detection is affected by sample content, volume of injection, solvent used and efficiency of the separation of the peaks. The extraction method may affect the LOD by the different parameters tested in the three- phase system e.g. pH of the donor- and acceptor phase and organic solvent. The presence of unwanted particles and other objects in the sample may also affect the LOD.
LOD is determined by measuring the signal- to – noise ratio(S/N) in the sample with a given concentration. Limit of detection is defined as S/N≥ 3. S is the height of the signal and N is the height of the noise.

$$\text{LOD} = \text{concentration of analyte in the sample (ng/1,1L)} \times 3 \times \frac{\text{Noise (cm)}}{\text{Signal (cm)}}$$

Limit Of Quantification (LOQ) is defined as the lowest concentration analyte that could be determined with an acceptable precision. It is a compromise between the concentration and the precision. The analytical results usually differ more widely when the analyte concentration is low. LOQ is determined the same way as LOD, but the quantification limit is defined as S/N ≥ 10[18].

$$\text{LOQ} = \text{concentration of analyte in the sample (ng/1,1L)} \times 10 \times \frac{\text{Noise (cm)}}{\text{Signal (cm)}}$$

3.7 Sewage treatment plants

Waste water treatment usually consist of three main steps; physical, chemical and biological cleaning. The sewage treatment plant (STP) Langnes, Tromsø, where samples have been collected consist only of physical cleaning. Physical cleaning together with chemical and biological cleaning is the preferred method.

The only barrier between the sewage and the outlet of the sewage treatment plant at Langnes is a 300 µm filter. During the filtration a huge quantity of sludge is obtained and delivered to composing. Physical cleaning of sewage water does not clean the outlet sewage water hundred percent, but measurements of samples taken from the outlet of the STP regarding outlet of sewage into the environment do not cross the regulations of permitted waste.

STP Lagnes receives waste water from households and business segments[20]. One section of the universital hospital in Tromsø, Åsgård, is also connected to Langnes sewage treatment plant. Benzodiazepines are widely used as sedatives at hospital awards like this. The connection of a hospital award could influence the amount benzodiazepines found in the sewage water[21].

Picture 1: Sewage treatment plant, Langnes, Tromsø

AIM OF THE THESIS

The aim of this thesis was to develop and optimize a Hollow Fiber- Liquid Phase MicroExtraction (HF-LPME) method for extraction and pre- concentration of benzodiazepines and metabolites in sewage water. The method should be optimized by performing HF-LPME with different organic solvents, essential oils, volatile oils, food oils, bases and acids, pH and hollow fibers. There has to my best knowledge never been performed analysis of Benzodiazepines in the environment by HF- LPME.

The optimized method should be performed on sewage water collected at Langnes STP in Tromsø.

4 MATERIALS AND METHODS

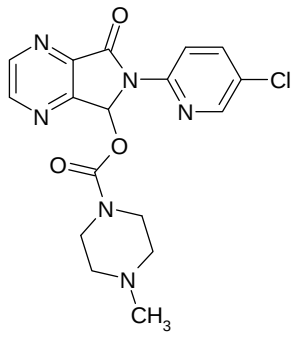
Chemicals

Zopiclone (4-Methyl-1-piperazine-carboxylic Acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]pyrazin-5-yl Ester), Zopiclone-d8 (4-Methyl-1-piperazine-d8-carboxylic Acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo[3,4-b]pyrazin-5-yl Ester) , Zopiclone N-Oxide (1-Piperazine-carboxylic Acid 4-Methyl-4-oxide 6-(5-Chloro-pyridin-2-yl)-7-oxo-6,7-dihydro-5H pyrrolo[3,4-b]pyrazin-5yl Ester), N-desmethyl Zopiclone Hydrochloride (Piperazine -1-carboxylic Acid 6-(5-Chloro-pyridin-2-yl)-7-oxo-6,7-dihydro-5H-pyrrolo[3,4-b]pyrazin-5yl Ester) , Zolpidem (N,N,6-Trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyrimidine-3-acetamide) , Zolpidem-d6 (N,N,6-Trimethyl-d6-2-(4-methylphenyl)imidazo[1,2-a]pyrimidine-3-acetamide), Alprazolam (8-Chloro-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine), Alprazolam 5-Oxide(8-Chloro-1-methyl-6-phenyl-4H-s-triazolo[4,3-a][1,4]benzodiazepine 5-Oxide), 1-hydroxy Alprazolam (a-Hydroxyalprazolam), Midazolam (8-Chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine), Midazolam –d5 (8-Chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine-d5) and 1`-hydroxy Midazolam (a-Hydroxymidazolam; 8-Chloro-6-(2-fluorophenyl)-1-hydroxymethyl-4H-imidazo[1,5a][1,4]benzodiazepine) were purchased from Toronto Research Chemicals (TRC, Toronto, ON, Canada). Clonazepam(50[2-Chlorophenyl]-1-3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one) and 7-Aminoclonazepam (7-Amino-5-[2-chlorophenyl]-1-3-dihydro-2H-1,4-Benzodiazepin-2-one)were purchased from Lipomed AG (CH-4144 Arlesheim, Switzerland).

Solvents for UPLC were Acetonitrile, isocratic grade for liquid chromatography (Merck, Darmstadt, Germany), Formic acid 98-100% pro analysi (Merck), Methanol(Merck) and water from a MilliQ purification unit from Millipore(Bedford, Massachusetts,USA).

Solvents for the hollow fiber liquid phase microextraction were; 3-octanol (Fluka AG, Buchs SG, Switzerland), 1-octanol (Sigma-Aldrich, Steinheim, Germany), Dihexyl ether (Sigma-Aldrich). Corn oil, Sesame oil(Naturata), Rape(seed)oil, cold-pressed(Odelia) and Sunflower oil were all purchased from Coop, Norway.

The essential oils; Almond oil (Pharmacy production, Oslo, Norway) and Peanut oil(Pharmacy production) and the volatile oils; Peppermint oil(Pharmacy production), Eucalyptus oil(Pharmacy production).
Sodium hydroxide pellets, GR for analysis (Merck). Formic acid 98-100% pro analysi(Merck), Ammonia Solution 32% (Merck), Hydrocholric acid fuming 37% (Merck).Tap water and water from a MilliQ purification unit from Millipore.



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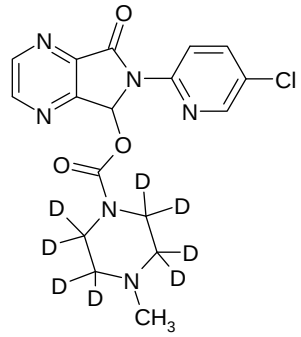

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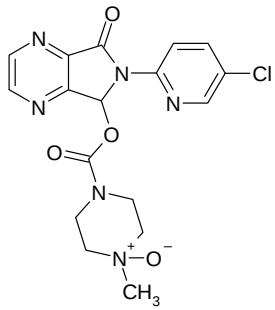

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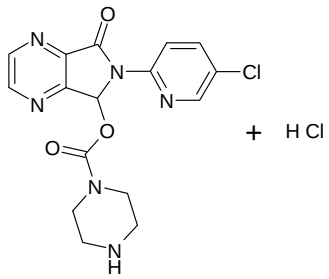

N-Desmethyl Zopiclone Hydrochloride
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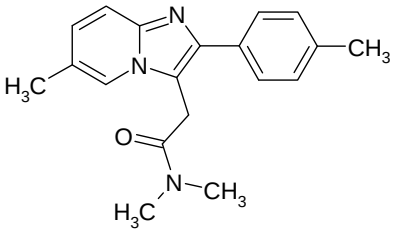

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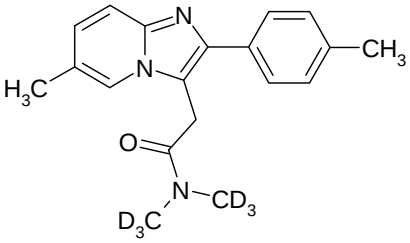

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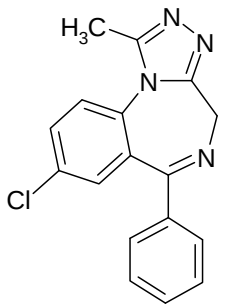

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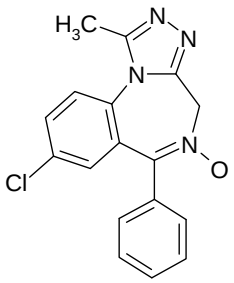

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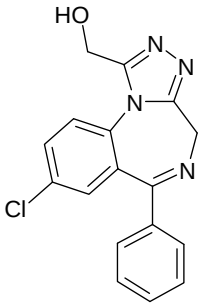

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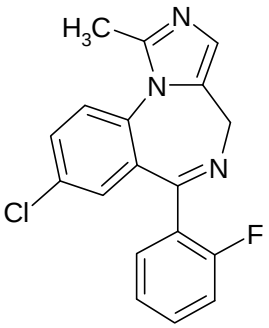

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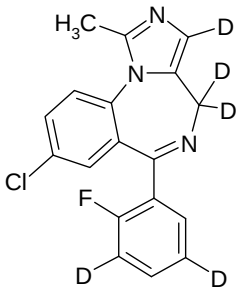

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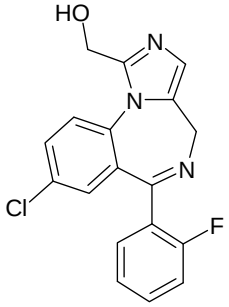

1-Hydroxy Midazolam
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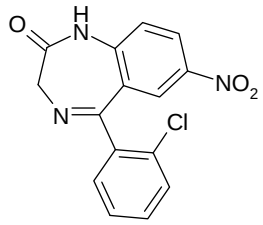
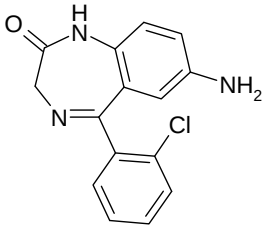
pKa= 4,39±0,40
CAS reg.nr.:59468-90-5



Clonazepam
Mon.masse: 315,04
pKa=1,55±0,25
CAS reg. nr.:1622-61-3



7-Aminoclonazepam
Mon.masse:285,07
pKa=3,92±0,40
CAS.reg.nr.:4959-17-5

Figure 5: Structure, pK_a- value, monoisotopic masses and CAS reg.no. of the chemical standards.

Preparation of Stock solutions and real samples

Stock solutions of each benzodiazepine and metabolite were prepared separately by dissolving proper amounts of each drug in HPLC grade methanol in concentrations of 50 or 100µg/mL. The Stock solutions were stored dark at -18 ºC.

Further, each Stock solution were diluted with a solution consisting of Millipore water (50 %), formic acid (0,1 %) and methanol (49,9 %) reaching a concentration of 1µg/ml of each compound. This diluted solution was used to tune the MS/MS parameters.

Further, a mixed Stock solution was prepared (25ng/mL) by diluting proper amounts of each Stock solution in Millipore water. This dilution was used in the UPLC-MS/MS method development.

Sampling Location

Wastewater samples were collected at Langnes STP, Tromsø, Norway. This sewage treatment plant uses an automatic sampling system to collect sample over a given period of time (picture 2). This is done to ensure that the collected sample is representative of the total amount sewage over a period of 24 ours.

Picture 2: Automatic sampling system of sewage water, Langnes (Tromsø).

5 Method development of Liquid Phase MicroExtraction

Previous studies using HF-LPME as an extraction method of different analytes has shown good results. Extraction of both large sample amount e.g. 1,1 L adjacent seawater and small amounts e.g. 0,2 - 1mL water, plasma and urine have previously been performed with success [15, 21, 22]. No articles have been found regarding HF-LPME of benzodiazepines in environmental samples.

The HF-LPME method developed for extraction of basic antidepressants by Terje Vasskog was used as a basis[21] and further optimized in order to perform the best extraction of the selected benzodiazepines.

Equipment

Medical syringe needles used during LPME; Terumo 0,5 x 16mm, 25G x 5/8" and
BD Microlance 3, 0,8 x 25mm, 21G x 1".

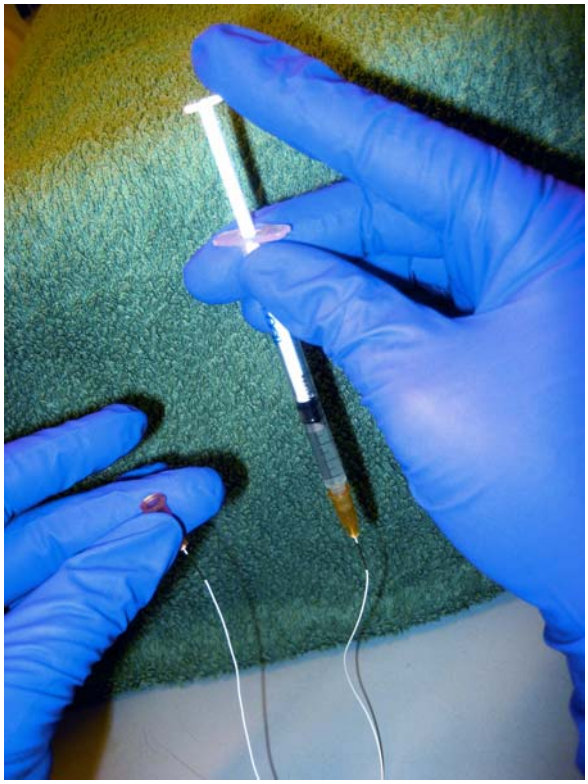
Hollow fibers used during LPME;

- Capillary Membrane (Type P1 LX), polypropylene: Inner diameter 330 µm, wall thickness 150 µm, bubble point 1,31 bar, transmembran flow 9,3 ml/[min x cm^2 x bar] and pore size max 0,47 µm.
- Hydrophobic capillary membrane(Type PP Q3/1), polypropylene: Inner diameter 600 µm, wall thickness 200 µm, bubble point 1,36 bar, transmembrane flow 2,1 ml/[min x cm^2 x bar] and pore size max 0,44 µm.
- Hydrophobic capillary membrane(Type PP Q3/2), polypropylene: Inner diameter 600 µm, wall thickness 200 µm, bubble point 0,95 bar, transmembrane flow 5 ml/[min x cm^2 x bar] and pore size max 0,63 µm.

All membranes were purchased from Membrana GmbH, Wuppertal, Germany.

Experimental

A hollow fiber was used to connect two medical syringe needles. The hollow fiber was dipped in an organic solvent, for about 20 seconds to fill the pores. Then an ultrasonic bath was used for about 3 seconds to remove excess solvent. After every third fiber, the water in the ultrasonic bath was changed. A 1 mL syringe was then attached to one of the medical syringes and used to fill the lumen of the hollow fiber with acidic acceptor solution (picture 3). The medical syringe needles were then removed and the ends of the hollow fibre were closed by a thin copper wire.



Picture 3: Demonstration of how to add acceptor solution into the lumen of the hollow fibre.

The HF- LPME was carried out in a 1000 ml glass bottle. 1,1 L tap water were added a given concentration of the analytes and made basic.
Screw cap with septum was used to close the bottle. A 0,8 mm syringe was inserted through the septum, and a thin metal wire was used to hold the fiber down in the water during the extraction (picture 4). The extraction was carried out with stirring at 800 rpm for 2 hours.

After extraction the acceptor solution was collected in a UPLC autosampler vial. This was done by attaching a 1 mL syringe with a medical syringe needle to one end of the fiber before air was used to push the acceptor phase through the fiber into the vial. The acceptor solution was directly injected to the LC-MS/MS. Each fiber was only used once.

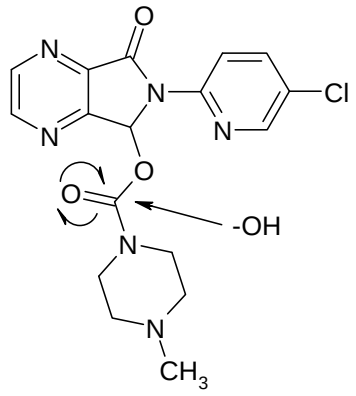
Picture 4: LPME carried out at 800 rpm for 2 ours.

Optimization of method

In order to optimize the method different organic solvents, essential oils, volatile oils, food oils, acides and bases, pH in the acceptor and donor phase, and 3 types of fibers were tested.

5.1.1 Hydrolysis of Zopiclone and metabolites

During the beginning of the method development it was discovered that zopiclone, zopiclone-d8, N-desmethyl zopiclone and zopiclone N-oxide were not detected on the MS during the analysis, after extraction. Because of the molecular structure of zopiclone and its metabolites we suspected basic hydrolysis of the molecule. See figure 6 for suspected point of attack. To confirm this, zopiclone, zopiclone- d8 and N- desmethyl zopiclone were added to three different solutions; water adjusted to pH 2 with formic acid, water adjusted to pH 10,5 with ammonia and one solution of pure Millipore water. The three samples were then injected to the UPLC-MS/MS. Zopiclone N- oxide was hard to detect on the MS, it was therefore excluded from this method development.



Zopiclone

Figure 6: Molecular structure of zopiclone and suspected point of attack, during basic hydrolysis.

After indications in the chromatograms that basic hydrolysis occurred, the solution with water adjusted to pH 10,5 with ammonia was infused directly into the MS to obtain a full scan.

The molecular weight of the products made after hydrolysis of zopiclone, zopiclone- d8, N-desmethyl zopiclone and zopiclone N-oxide was calculated and searched for in the full scan. Zopiclone and its metabolites should survive the acidic pH in the stomach, and acidic

hydrolysis of the molecules is therefore not suspected. This analytes contain some nitrogen's that are suspected to protect the molecule from undergoing acidic hydrolysis.

5.1.2 Extraction using different organic phases

Previously performed HF-LPME extractions have given good results using different organic phases. Plant oils, essential oils and traditional LPME solvents (dihexyl ether, n- octanol and dodecyl acetate) have all previously been used as intermediate extraction medium from different aqueous samples (water, plasma and urine) with a volume of 0.2 to 1 mL [22].
The traditional LPME solvents, dihexyl ether and 1- octanol have previously also been tested on large samples waste water and adjacent seawater(1,1 L) [16].
Essential oils and plant oils have the advantage of lower costs than the traditional LPME solvents. They are safer to work with and do not have to be disposed with special care.
In order to optimize the developed method for extraction of benzodiazepines using HF-LPME, different organic phases like traditional LPME solvents, 1- octanol, 3- octanol and dihexyl ether were tested. These traditional organic solvents were also replaced by essential almond oil and peanut oil. Two volatile oils; peppermint oil and eucalyptus oil, and plant oils used for cooking; corn oil, sesame oil, rape (seed) oil and sunflower oil was also tested as organic phases. Sesame oil and peanut oil has to my best knowledge never been used as organic phase during extraction with HF- LPME.
The extraction was carried out as previously described, using water adjusted to pH 12.65 with sodium hydroxide as donor phase and water adjusted to pH 2 with formic acid as acceptor phase. These conditions were chosen because of good results during extraction of selective serotonin reuptake inhibitors in environmental samples [21]. Three parallels of each concentration 250, 100 and 50 ng analytes were extracted and injected to the LC- MS/MS.

5.1.3 Extraction using different bases as donor phase

HF-LPME was not a successful extraction method for all the selected analytes. Clonazepam, 7-amino clonazepam, alprazolam and 1-hydroxy alprazoalm were not extracted using water adjusted to pH 2 with formic acid as acceptor phase and water adjusted to pH 12.65 with sodium hydroxide as donor phase. These mentioned analytes have lower pKa- values than the analytes that successfully were extracted. Therefore it was suspected that the analytes were not sufficiently basic, in order to be transported through the organic phase.
Because of this it was decided to try two different bases as donor phase; water adjusted to pH 13.95 with sodium hydroxide and water adjusted to pH 11 with ammonia.
The extraction was carried out as previously described, water adjusted to pH 2 with formic acid was used as acceptor phase and dihexyl ether was used as organic phase.
Three parallels of each concentration, 250, 100 and 50 ng analytes were extracted and injected to the LC-MS/MS.

5.1.4 Extraction using different acids as acceptor phase

Clonazepam, 7-amino clonazepam, alprazolam and 1-hydroxy alprazoalm were not detected on the MS/MS after extraction using water adjusted to pH 2 with formic acid as acceptor phase, dihexyl ether as organic phase and water adjusted to pH 12.65 with sodium hydroxide as donor phase.
It was suspected that some of these analytes were not properly ionized in the acceptor phase which leads to poor extraction. It was therefore decided to try two different acids as acceptor phase, water adjusted to pH 1 and pH 2 with hydrochloric acid and water adjusted to pH 2 with formic acid. The extraction was carried out as previously described using water adjusted to pH 12.65 with sodium hydroxide as donor phase and dihexyl ether as organic phase.
Three parallels of each concentration, 250, 100 and 50 ng analytes were extracted and injected to the LC-MS/MS.

5.1.5 Optimized method performed on different hollow fibers

The preferred method using 10 ml 5 M sodium hydroxide in 1,1 L water as donor phase (pH 12.65), dihexyl ether as organic phase and water adjusted to pH 2 with formic acid as acceptor phase was used to test different types of hollow fibers. The tested hollow fibers are made of the same material but the maximum pore size (µm) and other parameters e.g. wall thickness and diameter differed.

Until now fiber P1 LX has been used to perform the extractions using different organic phases, donor phases and acceptor phases. It was decided to try two different fiber types in order to optimize the method, and determine which fiber performs the best extraction of the selected analytes.

The tested fibers PP Q3/1 and PP Q3/2 had twice the length of fiber P1 LX. In order to fit into the 1000 ml glass bottle these fibers were divided into two equal- sized parts, 27 cm. The extraction was carried out as previously described. 7 parallels containing 50 ng analytes were extracted and injected to the LC-MS/MS.

5.1.6 Acidic hydrolysis of alprazolam, 1- hydroxy alprazolam, clonazepam and 7- aminoclonazepam?

Ugland et al. has previously performed LPME extraction of some selected weakly basic benzodiazepines from whole blood. According to this article some of their investigated benzodiazepines (nitrazepam, alprazolam, N- desmethyldiazepam and diazepam) undergo acidic hydrolysis in an aqueous solution. This hydrolysis is creating long- or short- lived intermediates (ring opening) see figure 10. The intermediates further degrade to the end product benzophenone[23].
During the method development for extraction of the selected benzodiazepines four analytes were not extracted; alprazolam, 1- hydroxy alprazolam, clonazepam and 7- aminoclonazepam. After reading this article it was decided to dissolve these analytes in three different solutins; pure Millipore water, water adjusted to pH 2 with formic acid and water adjusted to pH 10,5 with ammonia. In order to exclude basic hydrolysis of clonazepam and 7- aminoclonazepam the analytes were added to the basic solution. The three solutions were then injected to the UPLC-MS/MS.

Even though the analytes in all three solutions were detected on the MS it was decided to obtain a full scan of the solution made with water adjusted to pH 2 with formic acid. It was suspected to find some of the hydrolyzed products in the solution because of the obtained equilibrium between the analytes and there product.
The protonated molecular ion 327 and 343 were suspected to be found for alprazolam and 1- hydroxy alprazolam.
The protonated molecular ion 334 and 304 (clonazepam and 7- aminoclonazepam) were also searched for in the obtained full scan. See figure 7.

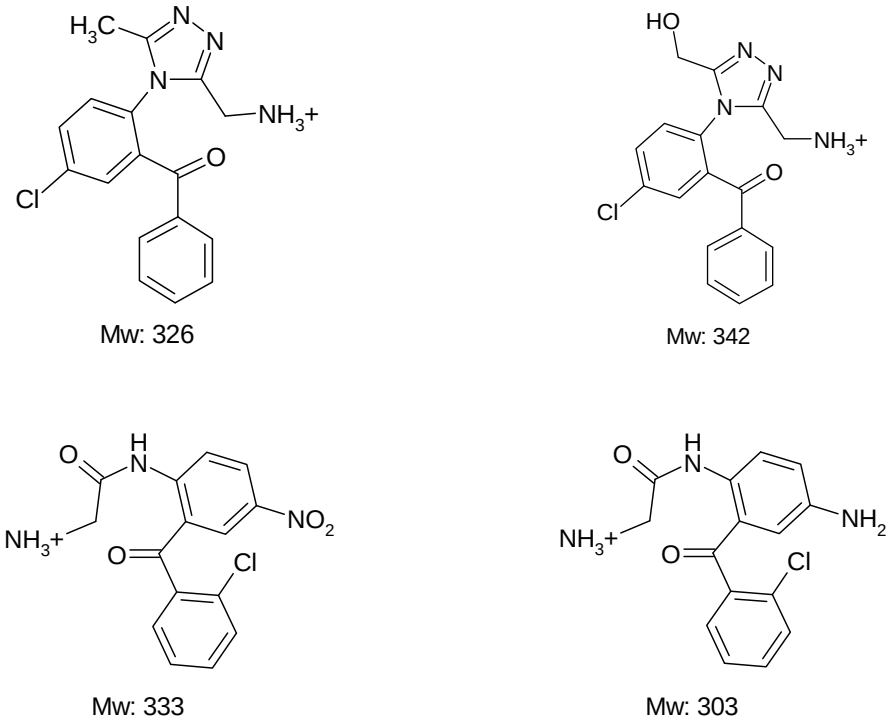


Figure 7: Molecules searched for in the obtained full scan.

6 MS/MS- analysis

6.1 Equipment

The detection was carried out on a Quattro Premier XE, Waters (Milford, MA,USA).
Sofware: Masslynx XT (Micromass)
Hamilton 1725 N (ga 22S/51 mm/pst 3), 250 µl Syringe (Hamilton Company, Reno Nevada, USA)

6.2 Experimental

Each compound was infused separately by a Hamilton syringe (250 µl) to the MS for detection of the molecular and product ions.
The *m/z* signal was optimized by changing the Cone Voltage until the signal was considered optimal. Cone Voltage was increased with increments of 5 V and fine-tuned until the optimal signal was found. All other terms were kept constant (see table 1). For detection of the product ions, MS/MS-scan was performed. Collision Energy (CE) was varied until optimal fragmentation was obtained. Se table 2 for product ion, CV and CE.

Table 1: Optimal terms for MS and MS/MS- analysis

Terms	Single MS	MS/MS
Flow(µl/min)	20	20
ESI- mode	Positive	Positive
Capillary (kV)	3,30	3,30
Cone (V)	Se table 2	Se table 2
Extractor (V)	2	2
RF lens (V)	0,2	0,2
Temp source (ºC)	100	100
Temp desolvetion (ºC)	250	250
Cone gas flow (L/hr)	20	20
Desolvation gas flow(L/hr)	100/200*	100/200*
LM Resolution 1	15	15
HM Resolution 1	15	15
Ion energy	1	1
Entrance	30	0
Collision	2	Se table 2
Exit	30	0
LM Resolution 2	14,5	14,5
HM Resolution 2	14,5	14,5
Multiplier	650	650

* 200 L/hr used during UPLC- MS/MS

Table 2: Dwell, optimal CV and CE for the protonated molecular ions and product ions.

	$[M+H]^+$	Product ion	CV	CE	Dwell
Clonazepam	316,19	270,03	38	23	0,5
7-Aminoclonazepam	286,19	250,20	39	20	0,5
Zoplidem	308,31	235,11	48	33	0,5
Zolpidem- d6 (IS)	314,31	235,04	52	38	0,1
Alprazolam	309,21	281,10	48	25	0,5
1-Hydroxy Alprazolam	324,23	295,18	42	25	0,5
Alprazolam 5-Oxide	325,00	205,00	34	27	-
Midazolam	326,25	291,08	52	27	0,5
Midazolam- d5 (IS)	331,29	296,25	56	27	0,1
1-Hydroxy Midazolam	342,25	323,98	45	20	0,5
Zopiclone	389,23	245,18	22	18	-
Zopiclone- d8 (IS)	397,10	245,00	20	18	-
Zopiclone N-oxide	405,05	143,20	26	15	-
N- desmethyl Zopiclone Hydrochloride	375,30	244,94	20	20	-

The mass spectra obtained of alprazolam 5-oxide and zopiclone N-oxide was poor, it was not possible to find the best fragment ion. These analytes were therefore excluded from the further method development.

7 Separation by UPLC

7.1 Equipment

The analysis was carried out by a Wates nano Acquity Ultra Performance LC. Chromatographic separation was performed by a reverse phase, acquity UPLC® BEH C_{18} 1,7µm, 1,0x150mm column from Waters.

7.2 Experimental

Protonated molecular ion, product ion, CV and CE, detected during MS/MS-analysis, were set up in the Multiple Reaction Monitoring (MRM) method of Masslynx. A mixture of all analytes, in known concentrations, was injected to the UPLC- MS/MS. The samples were injected in a volume of 5µl. The retention time for each compound was found and optimized by testing different gradients of the mobile phase. The optimal gradient was chosen and the retention time for each compound was plotted in to the MRM method. The MRM parameters used were kept constant (see appendix 14.2). The optimal situation is when the gradient separates the analytes properly so that the MS only focuses on a small number of analytes in each selected retention window (see table 4). In these retention windows the MS is focused only on the specific protonated molecular ion and product ion, while all other are excluded. In a MRM method the transition between the protonated molecular ion and the product ion is monitored by rapid switching of the electric fields applied to the mass analyzer to study each transition in turn. The MRM method provides an optimal sensitivity and much lower noise than the Single Ion Monitoring (SIM) method. MRM can result in a lower limit of detection than can be achieved in SIM because of the higher degree of specificity in MRM. On the other hand SIM provides higher peak areas[24, 25].

The first mobile phase tested consisted of Millipore water (solution A) and acetonitrile (solution B), used in a linear gradient. This mobile phase was not found optimal for elution of the analytes in the mixture, tailing was observed at some of the chromatograms. After this observation it was decided to try another composition of the mobile phase.

The new mobile phases consisted of Solution A; Millipore water and 0,1 % formic acid and solution B; 90 % acetonitrile, 10 % Millipore water and 0,1 % formic acid.

The retention time for the analytes changed when formic acid was added to the solutions and the problem with tailing improved. The change in retention time was corrected in the MRM

method but the gradient was kept the same (table 3). Within 16 minutes all analytes were eluted (table 4).

Table 3: Line, optimal flow and gradient
Solution A; Millipore water : 0,1 % formic acid
solution B; 90 % acetonitrile : 10 % Millipore water : 0,1 % formic acid.

Minutes	Gradient % solution A:B	Flow (µL)	Line
0	80: 20	50	-
10	60: 40	50	6
20	20: 80	50	6

Table 4: Retention window for the analytes using the optimal gradient.

Analytes	**Retention window (min.)**
7-Aminoclonazepam	3 - 7
1-Hydroxy Alprazolam	7 – 9
Zolpidem- d6	7 – 9
Zolpidem	7 – 9
Midazolam	9 – 13
Midazolam- d5	9 – 13
1-Hydroxy Midazolam	9 – 13
Alprazolam	13 - 16
Clonazepam	13 - 16

8 Preparation of the sample before extraction

The optimized method was performed on sampled sewage water.
These samples were taken by an automatic sampling system.
The sewage water was then measured into 1,1 L samples and added a given concentration of the internal standards, zolpidem- d6 (25 ng) and midazolam- d5(100 ng) . 10 ml 5 M sodium hydroxide was also added to make the sample basic. By adding a known concentration of internal standards, calculation of the concentration of unknown analytes could be performed by using the extracted standard curves.
The sample was then filtered through two glass microfiber filters (particle retention: 1,2 µm). This filtration removed particles and other unwanted objects from the water. Filtration may reduce the results of the amount analytes found in the sample, only the unbounded fraction of the analytes is measured. Due to a previous observation of Dr. Terje Vasskog it was decided necessary to filter the sewage water. During his work with HF- LPME extraction of basic analytes (SSRI`s) it was observed that a biofilm was developed on the outside of the fiber[21]. The biofilm was observed to slow down the extraction.

9 Optimized method performed on sewage water

A hollow fiber (Capillary Membrane, polypropylene, P1 LX) with an inner diameter of 330 µm, wall thickness of 150 µm, and max pore size 0,47µm, was used to connect two 0,5 x 16mm Terumo medical syringe needles(25G x 5/8”). The hollow fiber was dipped in an organic solvent, dihexyl ether, for about 20 seconds to fill the pores. Then an ultrasonic bath was used for about 3 seconds to remove excess solvent. After every third fiber, the water in the ultrasonic bath was changed. A 1 mL syringe was then attached to one of the medical syringes and used to fill the lumen of the hollow fiber with approximately 25µl acceptor solution (water adjusted to pH 2 with formic acid). The medical syringe needles were then removed and the ends of the hollow fibre were closed by a thin copper wire.
The HF- LPME was carried out in a 1000 ml glass bottle. Screw cap with septum was used to close the bottle. A 0,8 mm syringe was inserted through the septum, and a thin metal wire was used to hold the fiber down in the water during the extraction. The extraction was carried out with stirring at 800 rpm for 2 hours.
After the extraction the acceptor solution was collected in a UPLC autosampler vial. This was done by attaching a 1 mL syringe with a medical syringe needle to one end of the fiber before air was used to push the acceptor phase through the fiber into the vial. The acceptor solution was directly injected to the LC-MS/MS. Each fiber was only used once.

Extraction of a blank sample

A blank sample, sample without analytes, was extracted in order to confirm that the tap water used for extraction of the standard curves was not contaminated with benzodiazepines. The extraction was performed as previously described. 1,1 liter tap water was adjusted to pH 12.65 with sodium hydroxide (donor phase), dihexyl ether was used as organic phase and water adjusted to pH 2 with formic acid was used as acceptor phase.
Blank samples like this are used to ensure that the method is not influenced by the personal or chemicals present in the extraction method e.g. dihexyl ether, sodium hydroxide and formic acid.

Standard curves

Several of the selected benzodiazepines in this method development were not extracted using the optimized method. Zopiclone and its metabolites experienced basic hydrolysis and were therefore excluded from this method development. Alprazolam, 1-hydroxy alprazolam, clonazepam and 7-aminoclonazepam may not have been sufficiently ionized or been hydrolysed in the acidic acceptor phase.

The optimized method was used and performed as previously described in order to make a standard curve for; midazolam, 1- hydroxy midazolam and zolpidem.
The selected internal standards should have a similar behaviour as the analyte and are added in a known concentration in order to adjust for random alterations during the extraction.
1,1 L tap water added 10 ml 5 M sodium hydroxide (pH 12.65) was used as donor phase, dihexyl ether as organic phase and water adjusted to pH 2 with formic acid was used as acceptor phase. Midazolam- d5 (100 ng) and zolpidem- d6 (25 ng) was used as internal standards.
Each concentration of analyte 250- 100- 50- 25- 10 and 1 ng was extracted with 6 parallels in order to make a reliable standard curve.

Calculation of LOD and LOQ

The extraction of the standard curves, 6 batches, was used to calculate LOD and LOQ for the extracted analytes zolpidem, midazolam and 1- hydroxy midazolam.
1-hydroxy midazolam was not detected in the lowest concentration of 1 ng analytes, LOD and LOQ was therefore calculated from the extraction using 10 ng analytes.

The calculation was performed by measuring the height of the noise and the height of the signal, these measurements were put in to the following formulas used for calculation:

$$\text{LOD} = \text{concentration analyte(ng/1,1L)} \times 3 \times \frac{\text{Noise (cm)}}{\text{Signal (cm)}} = \text{X ng/1,1L}$$

$$\text{LOQ} = \text{concentration analyte (ng/1,1L)} \times 10 \times \frac{\text{Noise (cm)}}{\text{Signal (cm)}} = \text{X ng/1,1 L}$$

10 RESULTS AND DISCUSSION

10.1.1 Hydrolysis of Zopiclone and metabolites

HF-LPME is not a successful extraction method for zopiclone and its metabolites. The injection of the three solutions into the LC-MS/MS, revealed that during the extraction the ananlytes undergo basic hydrolysis (see figure 9). When injecting the sample with water adjusted to pH 10,5 with ammonia there were no signal of the molecules(see chromatogram 3). The sample made with Millipore water and the last sample of water adjusted to pH 2 with formic acid gave signal (see chromatogram 1 and 2). This underlines the assumption that a basic hydrolysis occur.
Structurally zopiclone and its metabolites have a similar ground structure, but they do have a side chain that differs from each other, R. See figure 8.

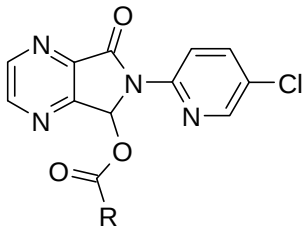


Figure 8: Ground structure of zopiclone and its metabolites

By using full scan, in the search for the different side chains (*m/z*), R, the MS was not able to separate zopiclone from its metabolites. Zopiclone and its metabolites were therefore excluded from the extraction method development.

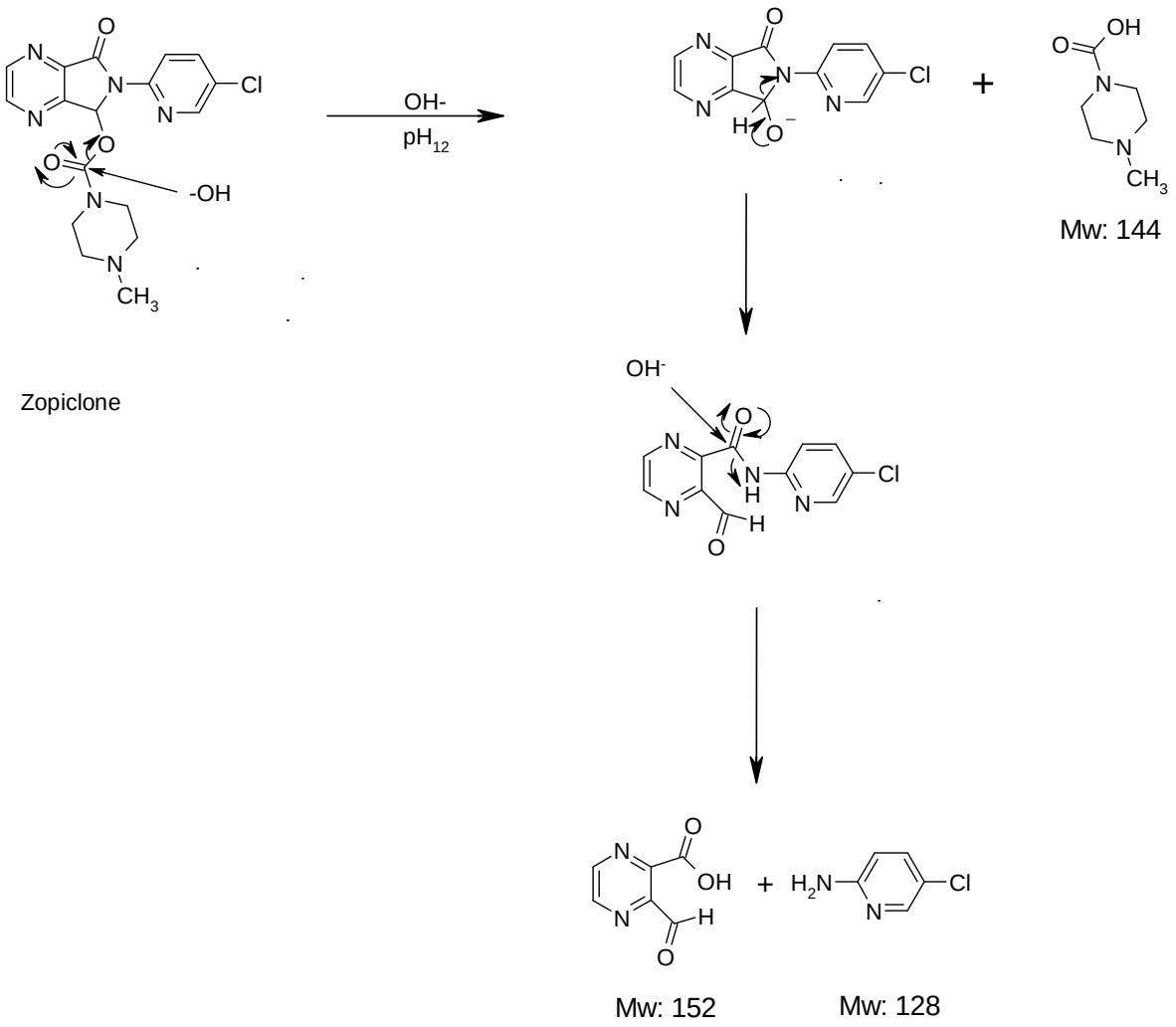
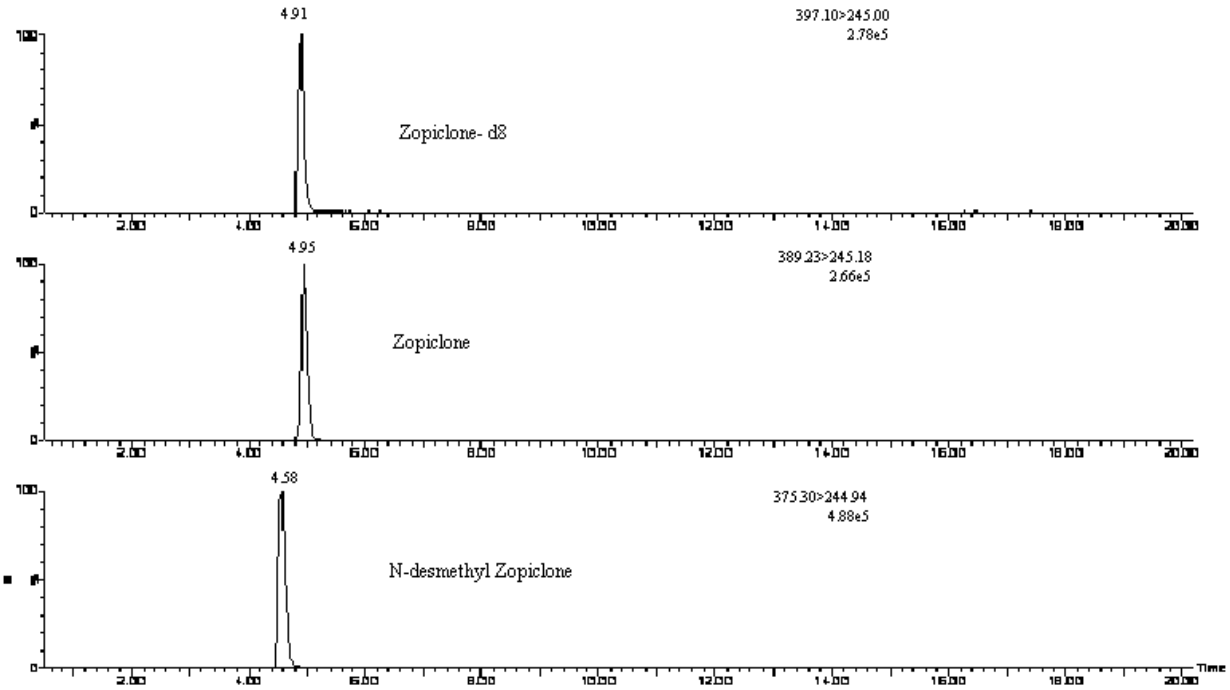
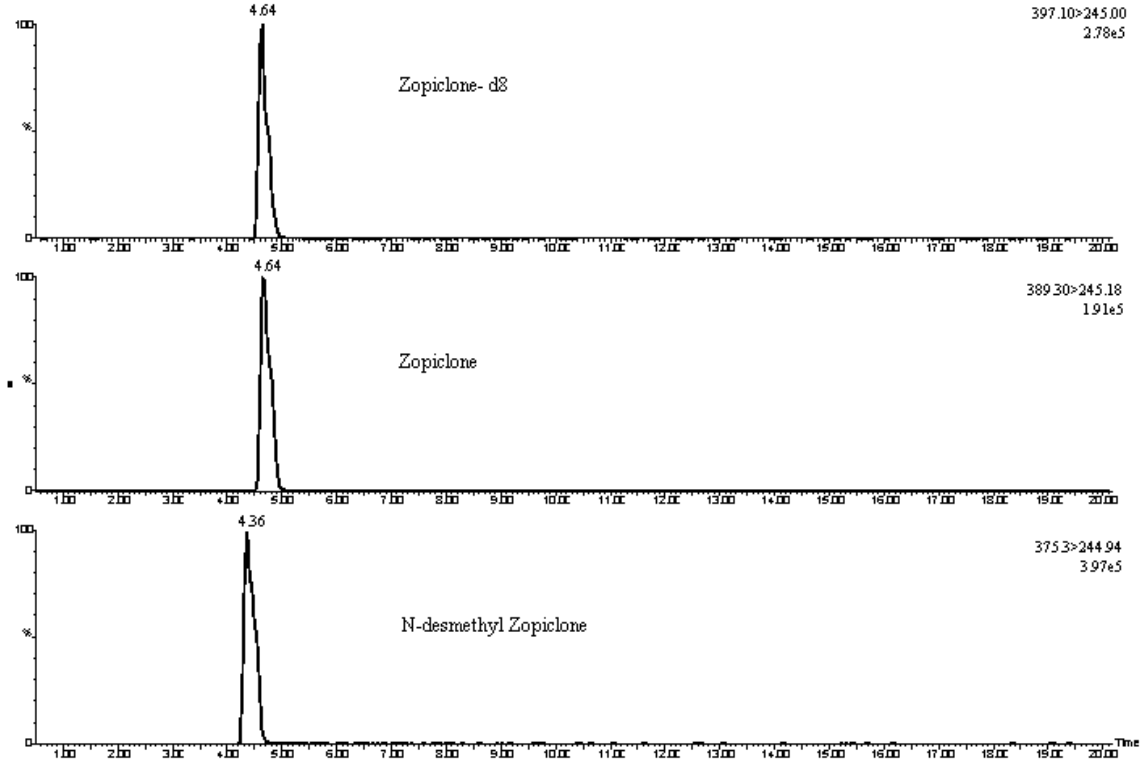


Figure 9: Basic hydrolysis of zopiclone. The same reaction occurs for its metabolites[26].

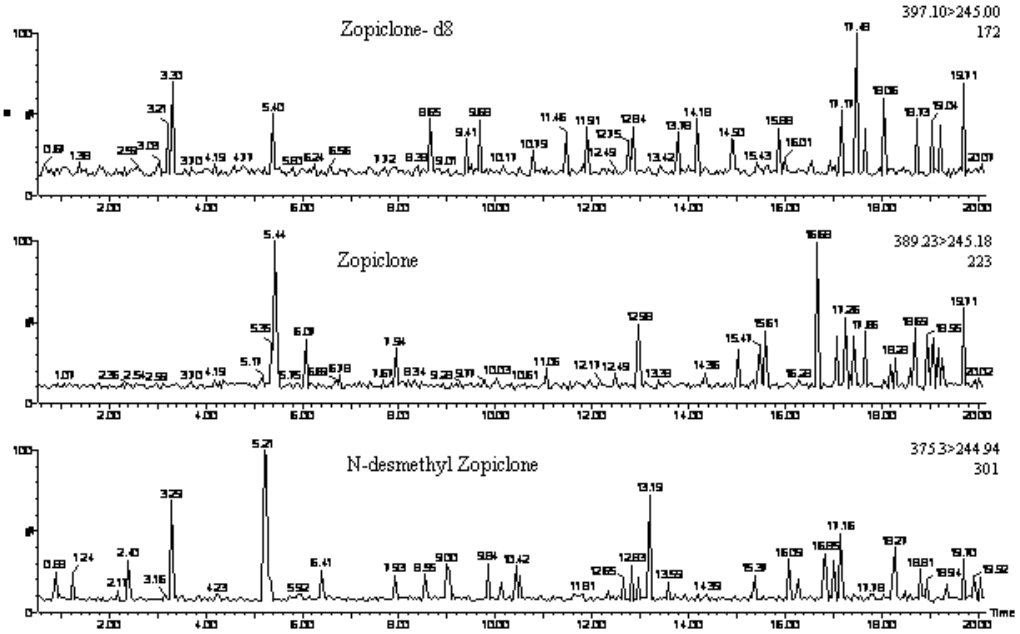
By searching for the different molecular ions made during the hydrolysis, protonated molecular ion 129 was detected within the full scan chromatogram (chromatogram 4). The protonated molecular ion 153, 145, 131 and 161 were not detected. This could be due to poor ionization in ESI^+ caused by the acidic group in the molecules (see figure 9).



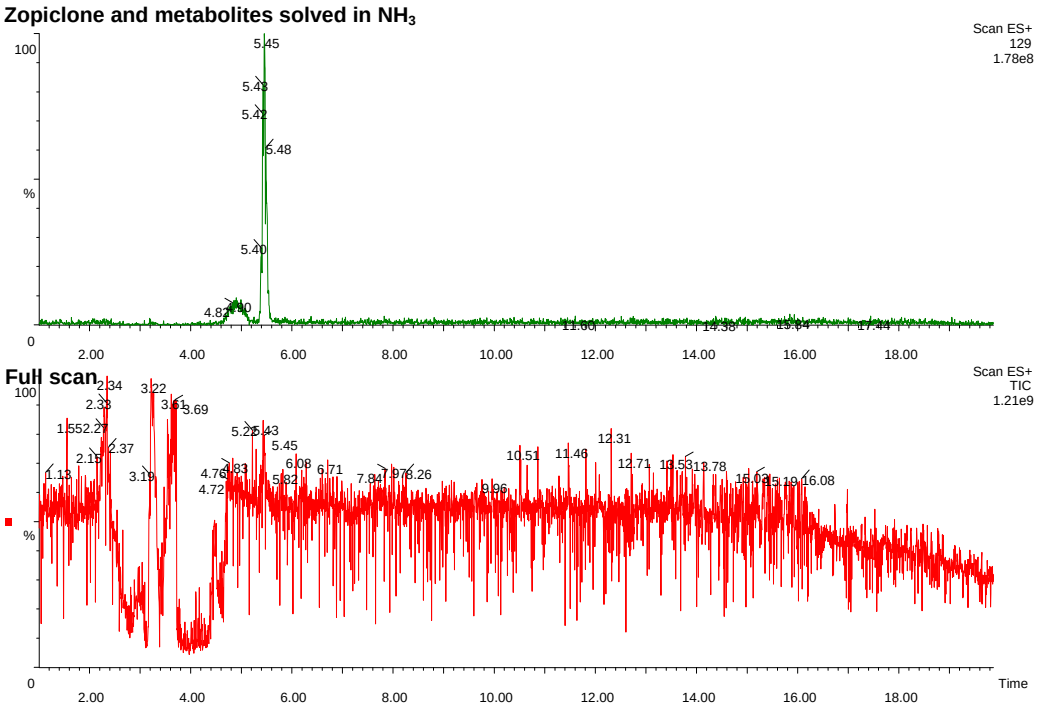
Chromatogram 1: Zopiclone, zopiclone- d8 and N-desmethyl zopclone dissolved in Millipore water.



Chromatogram 2: Zopiclone- d8, zopiclone and N-desmethyl zopiclone dissolved in water adjusted to pH 2 with formic acid.



Chromatogram 3: Zopiclone- d8, zopiclone and N-desmethyl zopiclone dissolved in water adjusted to pH 10,5 with ammonia.



Chromatogram 4: Protonated product ion 129 found in the obtained full scan. Zopiclone, zopiclone- d8 and N- desmethyl zopiclone dissolved in water adjusted to pH 10,5 with ammona.

10.1.2 Extraction using different organic phases

Extraction using different organic phases was carried out with varying results.

1- octanol, 3- octanol and Dihexyl ether used as extraction media

n- octanol and dihexyl ether are among the organic solvents traditionally used during LPME. These organic solvents are known to have good extraction qualities, low polarity and viscosity[22].

During extraction with 1- octanol, air bubbles occurred on the outside of the fibers. These air bubbles could indicate high stirring speed, or it could be a result of the large amount of sample extracted (donor phase). The organic phase could have been washed out into the sample. Without organic phase immobilized in the pores of the hollow fiber no extraction will occur.

Neither 3- octanol was suited as organic solvent. When air was pushed through the fiber for collection of the acceptor phase, there was no sample in the fiber. As the air was pushed through, the sample came out the walls of the fiber. This can indicate leakage of the organic phase into the donor phase due to long extraction time and poor immobilization in the pores of the hollow fiber.

The distribution coefficient (log- D) of the organic solvents affects the quality to perform extraction. The higher distribution coefficient the more hydrophobic is the solvent/molecule. 1- octanol has an log- D of 3 and 3- octanol 2,82 at 25ºC[27]. Dihexyl ether has a log- D value of 5,23 which is higher than for the two octanole solvents used. The difference in the log- D values of the selected organic solvents may participate in the poor extraction results using 1- and 3- octanol. A low log –D value of the organic phase will make it more hydrophilic which could result in wash out from the fibres into the sample.

For average area after extraction and chromatograms see appendix 14.3 and 14.4.

Volatile oils used as extraction media

Peppermint oil and eucalyptus oil performed poor extraction of the selected analytes. According to the producer of peppermint oil and eucalyptus oil, Pharmacy Production in Oslo, there may be some remains of solvents used during the production. Steam distillation, used for production of these volatile oils, uses some water soluble components e.g. ethanol. Some of these water soluble components may be found in the end product and influence the quality as organic phase during HF- LPME. High polarity of the organic phase could lead to leakage into the large sample amount and thereby perform poor extraction.
Peppermint oil and eucalyptus oil may not be retained with sufficient strength in the pores of the hollow fibre. The large sample amount (1,1L) and long time of extraction could intensify the leakage into the donor phase.
Peppermint oil and eucalyptus oil have previously successfully been used as organic phase in small sample amounts of water, plasma and urine (0,2 – 1 ml)[22].
For average area after extraction and chromatograms see appendix 14.3 and 14.4.

Essential oils used as extraction media

Both Almond and peanut oil performed HF-LPME successfully. 1-hydroxy midazolam, midazolam- d5, miadzolam, zolpidem and zolpidem- d6 were all extracted using these essential oils. The average area after extraction with almond oil and peanut oil were poorer than extraction performed with dihexyl ether. Dihexyl ether was therefore preferred.
For average area after extraction and chromatograms see appendix 14.3 and 14.4.

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Plant oils used for cooking as extraction media

Four plant oils usually used for cooking, sunflower oil, corn oil, sesame oil and rape(seed) oil were immobilized in the pores of the hollow fiber as organic phase. All four tested plant oils succeeded as organic phase. Unfortunately the area under the curve was lower than using dihexyl ether, one of the traditional solvents used in LPME.

Corn oil was the plant oil that performed the best extraction of the four tested oils. Rape (seed) oil was observed to perform the poorest extraction of the different plant oils tested. During the extraction work it was observed that Rape (seed) oil has a higher viscosity than Corn oil. High viscosity could influence and limit the diffusion rate of analytes across the organic phase.

Pedersen- Bjergaard and Rasmussen have suggested that hydrolysis of fatty oils may occur when the extraction time are prolonged over 45 minutes [22].

During the extraction of the selected benzodiazepines using plant oils it was observed that the polypropylene hollow fibers had a more spongy appearance after the extraction. This could indicate that a hydrolysis of the fatty oils (saponification) may have occurred in some degree and resulting in poorer extraction. The fatty oils used are in contact with the base for a longer time (2 hours) and the volume of basic donor phase are larger than the sample used in Pedersen- Bjergaards and Rasmussens extraction. These parameters may contribute to hydrolysis in some degree.Fatty acids could undergo saponification in contact with sodium hydroxide. The ester groups are hydrolyzed and produce glycerol and fatty acid salts (e.g. see figure 10).

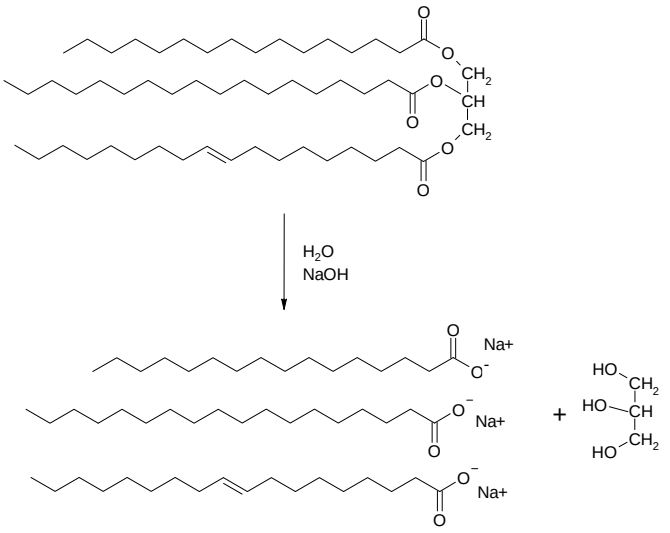


Figure 10: Saponification mechanism[28].

Dihexyl ether performed better extraction than the selected plant oils. The poorer extraction using plant oils may be caused by saponification or other extraction limiting factors like viscosity. For average area after extraction and chromatograms see appendix 14.3 and 14.4.

10.1.3 Extraction using different bases as donor phase

Clonazepam, 7- aminoclonazepam, alprazolam and 1- hydroxy alprazoalm were not extracted using water adjusted to pH 12.65 with sodium hydroxide as a donor phase. It was therefore decided to try different pH levels and different bases in the donor phase.
Extraction performed using water adjusted to pH 11 with ammonia and 100 ng analytes as the donor phase was no success. 1-hydroxy midazolam, midazolam- d5, midazolam, zolpidem and zolpidem- d6 were detectable in the sample but not quantifiable. Ammonia is a weaker base than sodium hydroxide, this may contribute to a lower performance of extraction. The sample might not have been sufficiently alkaline for extraction of the basic analytes.

Sodium hydroxide was used as donor phase with success.
Water adjusted to pH 12.65 with sodium hydroxide performed the HF-LPME extraction with the best result at the lowest added concentration (50 ng /1,1L) analytes. Water adjusted to pH 13.95 with sodium hydroxide as donor phase gave the best extraction results with concentrations over 100 ng analytes. To my best knowledge there has not been found concentrations higher than 50 ng /1,1L in sewage water samples. It was therefore decided to use water adjusted to pH 12.65 with sodium hydroxide as donor phase.
Clonazepam, 7- aminoclonazepam, alprazolam and 1- hydroxy alprazolam were not extracted using the selected bases and pH- levels.
For average area after extraction and chromatograms see appendix 14.3 and 14.4.

10.1.4 Extraction using different acids as acceptor phase

Acceptor phase using water adjusted to pH 2 with hydrochloric acid performed extraction of the analytes in some degree, but not consistent.
The extraction using water adjusted to pH 1 with hydrochloric acid extracted the analytes sufficiently and the area under the curve was consistent. The analytes were probably sufficiently ionized and therefore captured in the acceptor phase for detection. Hydrochloric acid is a stronger acid than formic acid but it was not found good enough to perform extraction of clonazepam, 7- aminoclonacepam, alprazolam and 1-hydroxy aloprazolam.
The average area after extraction was nearly the same using water adjusted to pH 1 with hydrochloric acid and water adjusted to pH 2 with formic acid.
Water adjusted with formic acid performed the extractions to some degree better than water adjusted to pH 2 with hydrochloric acid. This solution was there for found to be the optimal acceptor phase. Formic acid is a weaker acid than hydrochloric acid and considered more compatible with the mobile phase and the UPLC- MS system. For average area after extraction and chromatograms see appendix 14.3 and 14.4.

10.1.5 Optimized method performed on different hollow fibers

All three fibers performed the HF-LPME extraction with good results. Fiber PP Q3/2 performed best extraction for 1- hydroxy midazolam and midazolam.
Midazoalm- d5, zolpidem and zolpidem- d6 had best extraction using fiber P1 LX.
Fiber PP Q3/2 has a max pore size of 0,63 µm, which is higher than the rest of the fibers used in this method development. The different pore sizes of the fibers may influence the ability to extract. It may take longer time to immobilize the organic phase in the pores of the hollow fiber. It was noticed that the variations of results was greater within the two fibers PP Q3/1 and PP Q3/2. These high variations of the parallels may be caused by poor immobilization of organic phase in some fibers or the fact that the wall of the hollow fibers, PP Q3/1 and PP Q3/2, are thicker (200µm) than the little fiber P1 LX (150 µm).
The extraction time of two hours may not be sufficient in order to achieve equilibrium with a wall thickness of 200 µm this may also influence the consistency.
The lumen of fiber PP Q3/1 and PP Q3/2 hold a larger acceptor phase volume than fiber P1 LX. Fiber PP Q3/1 holds approximately 72 µl while fiber PP Q3/2 holds 33µm and P1 LX 25 µl. A large volume of acceptor phase injected to the LC- system could provide better LOD

and LOQ[17].

For average area after extraction and chromatograms see appendix 13.3 and 13.4.

10.1.6 Acidic hydrolysis of alprazolam, 1-hydroxy alprazolam, clonazepam and 7- aminoclonazepam?

The suspected hydrolysis of alprazolam, 1- hydroxy alprazolam, clonazepam and 7- aminoclonazepam are as follows:

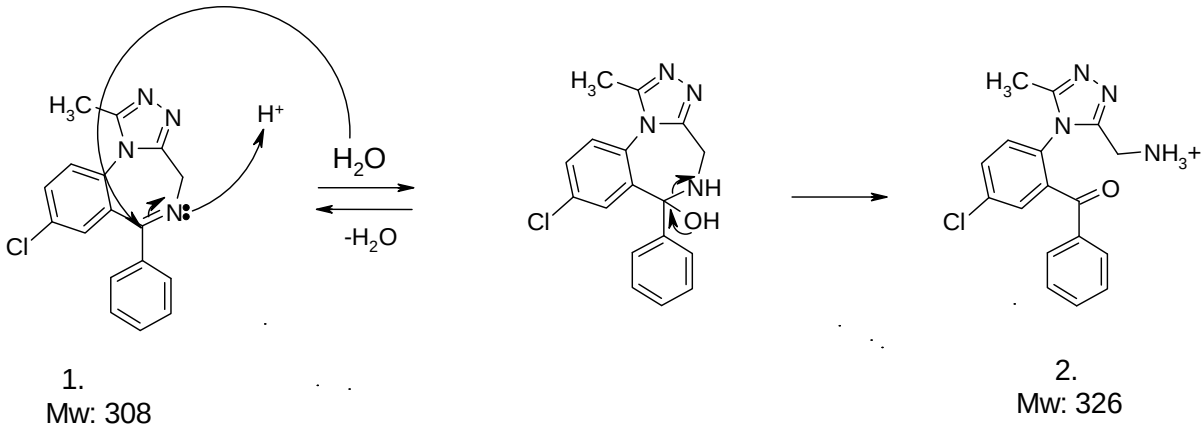
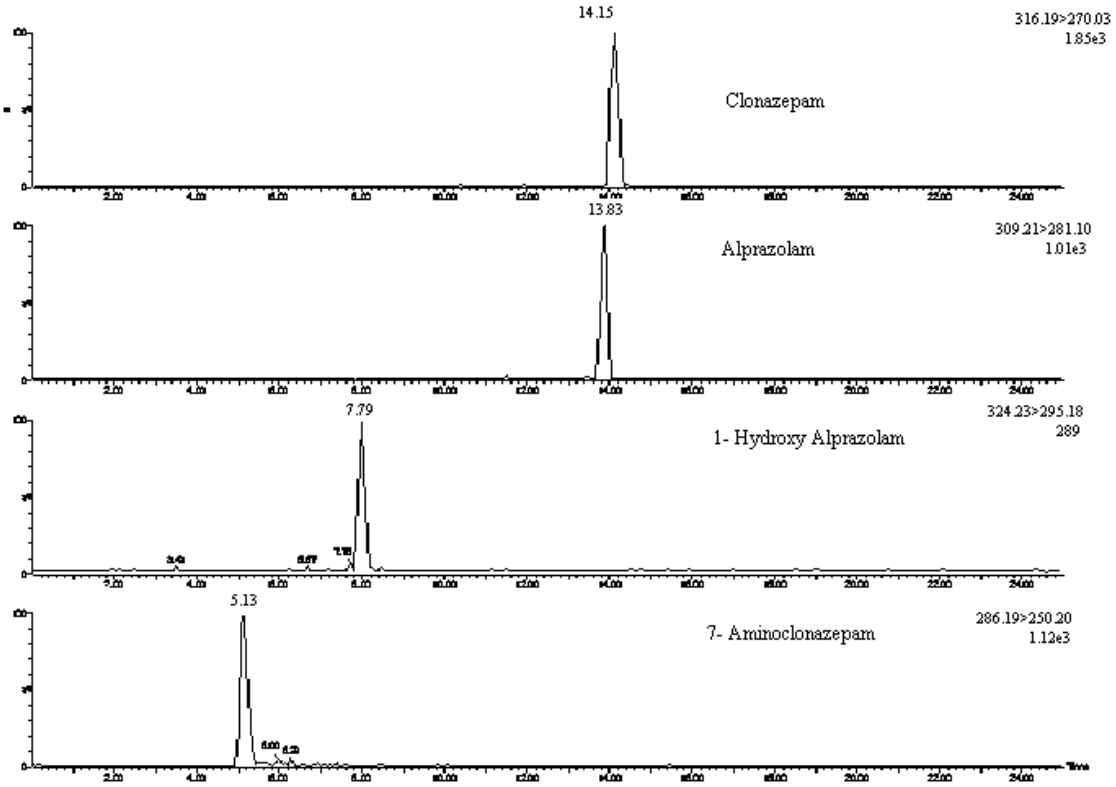


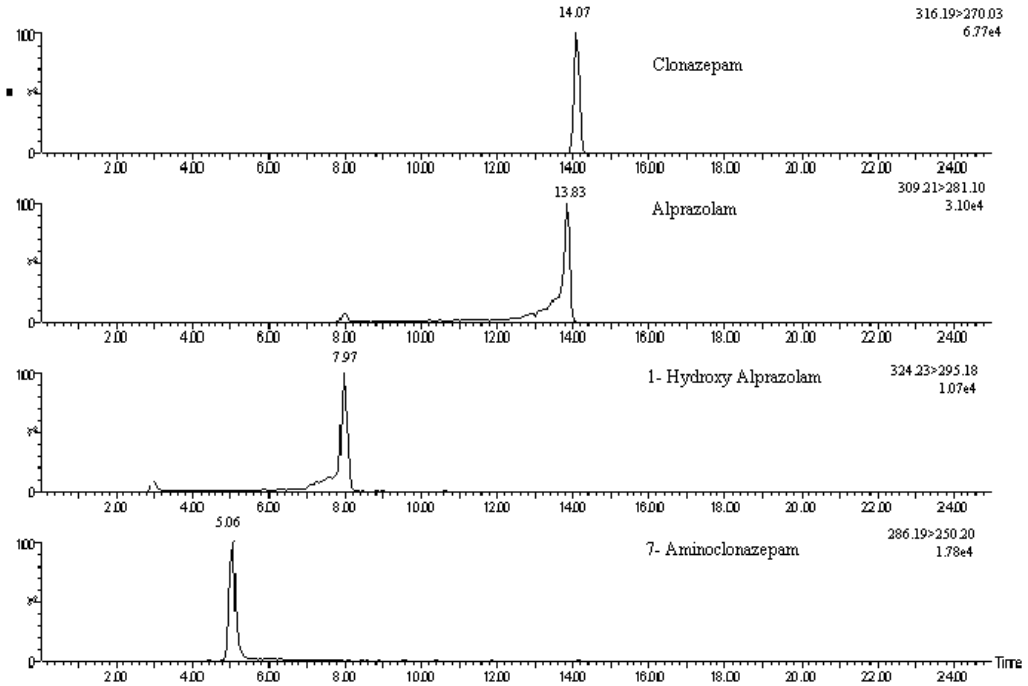
Figure 11: E.g. acidic hydrolysis of alprazolam[29].

The injection of the three solutions into the LC-MS/MS, revealed that no acidic or basic hydrolysis had occurred. It was possible to detect alprazolam, 1- hydroxy alprazolam, cloazepam, 7- aminoclonazepam in the solutions made of pure Millipore water, water adjusted to pH 2 with formic acid and water adjusted to pH 10,5 with ammonia. See chromatogram 5-7.

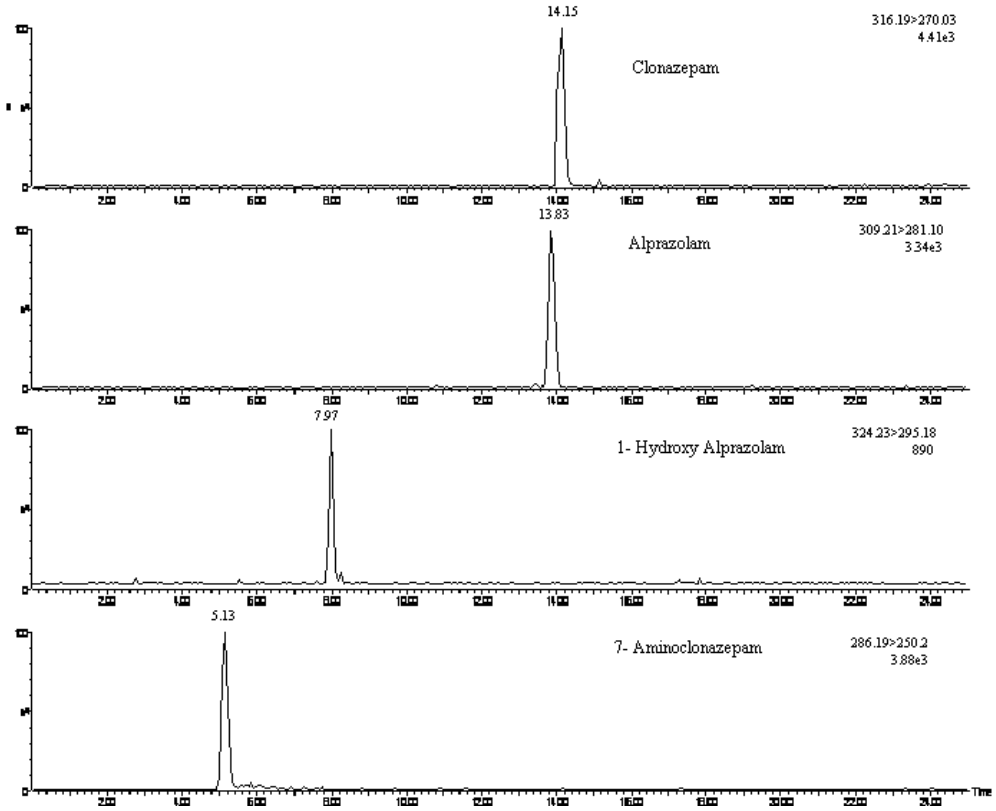
The mechanism of the acidic hydrolysis is in an equilibrium between molecule 1 and 2 (see figure 11). Because of the equilibrium it was decided to search for the protonated molecular ions of the suspected hydrolyzed compounds in the obtained full scan.



Chromatogram 5: Clonazeam, alprazolam, 1- hydroxy alprazolam and 7- aminoclonazepam dissolved in Millipore water.



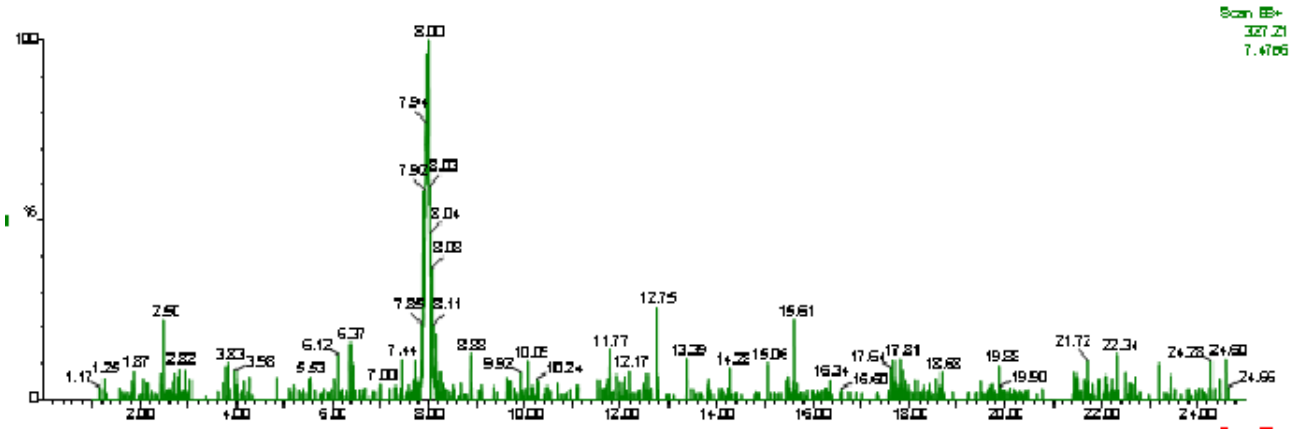
Chromatogram 6: Clonazepam, alprazolam, 1- hydroxy alprazolam and 7- aminoclonazepam dissolved in water adjusted to pH 2 with formic acid.



Chromatogram 7: Clonazepam, alprazolam, 1- hydroxy alprazolam and 7- aminoclonazepam dissolved in water adjusted to pH 10,5 with ammonia.

The searches for the suspected product ions after hydrolysis of alprazolam and 1- hydroxy alprazolam were detectable in the obtained full scan.

E.g. ion $[M+H]^+$ 327.21 for the hydrolyzed alprazolam product (according to figure 11) was detectable(see chromatogram 8).



Chromatogram 8: E.g. molecular ion 327.21 found in the obtained full scan. Alprazolam, 1- hydroxy alprazolam, clonazepam and 7- aminoclonazepam dissolved in water adjusted to pH 2 with formic acid.

Protonated molecular ions from hydrolyzed clonazepam and 7-aminoclonazepam were not found in the obtained full scan.

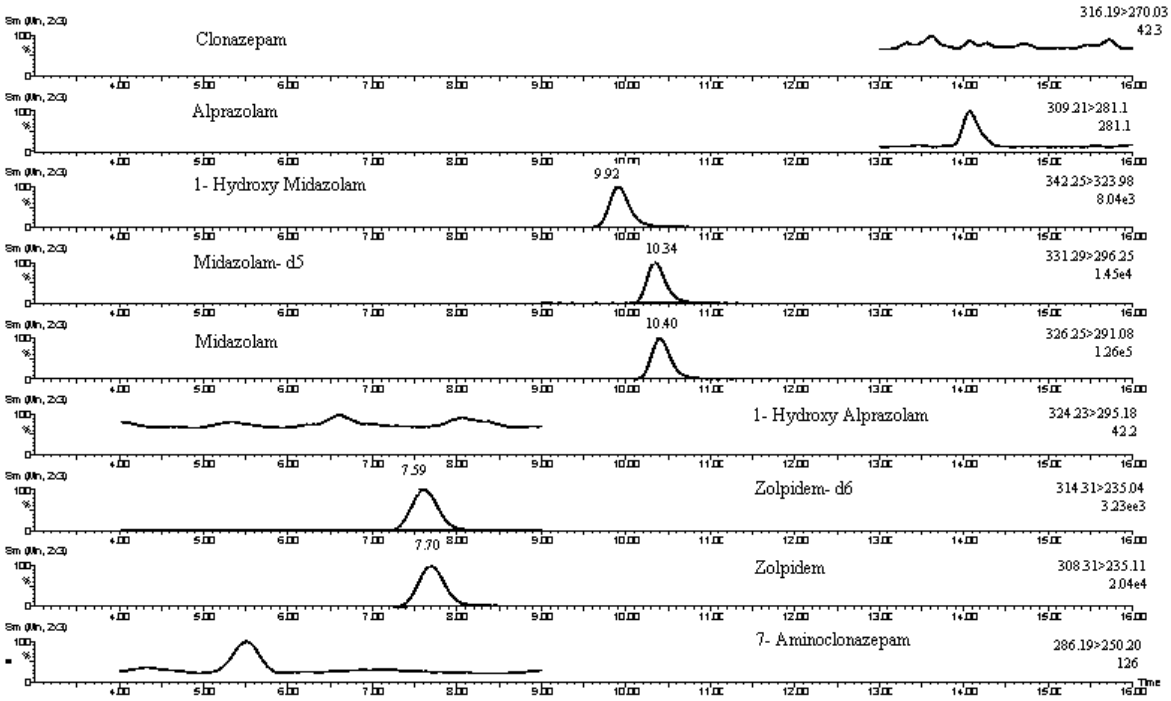
Low pK_a- values of alprazolam, 1- hydroxy alprazolam, clonazepam and 7- aminoclonazepam may also contribute to poor extractioon. These analytes may not have been sufficiently ionized to immobilise the analytes in the acceptor phase.
The acceptor phase should be at least 3.3 pH units below the pK_a of basic analytes to perform extraction[23].
Clonazepam, 7- aminoclonazepam, alprazolam and 1-hydroxy alprazolam has all pK_a – values below 4. The acidic capacity in the acceptor phase used may have been a limiting factor.

Extraction of a blank sample

The blank sample extracted did not reveal benzodiazepines in the water. This indicates that the method is not influenced by other chemicals like formic acid present in the extraction, and that the equipment and the personal do not contaminate the sample. See appendix 14.5.

Standard curves

The standard curves were plotted by calculating the ratio analyte over internal standard. This was then plotted against the concentration of added analyte. All three extracted standard curves were calculated to have a difference quotient of 0.99 (see appendix 14.1).
1- hydroxy midazolam was not extracted at the lowest concentration 1 ng/ 1,1 L. The lowest concentration for this analyte is therefore 10 ng/ 1,1L sample.



Chromatogram 9: MRM chromatogram after smoothing. 250 ng analytes added the donor phase.

Calculation of LOD and LOQ

Calculation of LOD and LOQ was performed as previously described.

Table 5: Limit Of Detection and Limit Of Quantification for the extracted analytes using HF-LPME. Water adjusted to pH 12.65 with sodium hydroxide used as base in the donor phase, dihexyl ether as organic phase and water adjusted to pH 2 with formic acid as acceptor phase.

Analytes	LOD	LOQ
Zolpidem	0,2 ng/1,1L	0,7 ng/1,1L
Midazolam	0,3 ng/1,1L	1,1 ng/1,1 L
1- Hydroxy Midazolam	4,7 ng/1,1 L	15,9 ng/1,1L

1- hydroxy midazolam has a higher LOD and LOQ- value than zolpidem and midazolam. The high LOD and LOQ- value indicate why 1- hydroxy midazolam not were detected in the concentration of 1 ng added analytes.

Extracted sewage water samples

Analyses of collected sewage water were performed in January and April.
The extraction performed in January had quantifiable results for zolpidem.
1- hydroxy midazolam and midazolam were detectable. For chromatograms see appendix 14.6.
In April none of the extractions performed had quantifiable results, but 1- hydroxy midazolam, zolpidem and midazolam were detectable. The difference in result between these months could be due to the high ice melting in April. The low amount of analytes found in the samples taken in April is therefore most truly due to dilution of the sewage by melt water.

Benzodiazepines are intended for short time use and the dosages administered are generally lower than for e.g. antidepressants that are usually intended for a longer period of time. Benzodiazepines are mostly prescribed in dosages of 5– 20 mg daily, while antidepressants usually are prescribed in dosages of 20- 200 mg depending on the activity of the drug and severity of the medical condition[30]. It is therefore suspected to find lower concentrations of benzodiazepines in environmental samples compared to antidepressants. Citalopram and sertralin are among the most prescribed antidepressants used in Norway. In Troms 2008 it was registered that 421479 defined daily dosages (DDD) citalopram had been used, 242094 DDD sertralin.
Zopiclone is the most prescribed benzodiazepine in Norway. In 2008 1226260 DDD of zopiclone were purchased from pharmacies in Troms. Unfortunately zopiclone is among the analytes that were not extracted using HF-LPME due to basic hydrolysis of the compound. Zolpidem are the second most prescribed benzodiazepine, 153378 DDD[31]. At Langnes STP it was possible to quantify the amounts zolpidem found (3 ng/ L sewage water) in samples taken in January. Based on sale statistic it is likely to find higher concentrations of zopiclone than zolpidem in environmental samples.

Table 6: Table of concentration, detectable (D) and not detectable (ND) analytes in samples collected and extracted in January and April.

Analytes	January	April
7-Aminoclonazepam	ND	ND
1-Hydroxy Alprazolam	ND	ND
Zolpidem- d6	Internal Standard	Internal Standard
Zolpidem	3 ± 2,7 ng / L waste water	D
Midazolam	D	D
Midazolam- d5	Internal Standard	Internal Standard
1-Hydroxy Midazolam	D	D
Alprazolam	ND	ND
Clonazepam	ND	ND

Zolpidem was the only quantifiable analyte found in the waste water collected at Langnes in January. The concentration was calculated to be 3 ± 2,7 ng /L waste water. The high standard deviation of the samples extracted (four parallels) could be a result of the low analyte concentration in the sample. The analytical results differ more widely when the concentration of analytes is low.

11 CONCLUSION

Extraction using water adjusted to pH 12.65 with sodium hydroxide as donor phase, water adjusted to pH 2 with formic acid as acceptor phase and dihexyl ether as organic phase became the preferred method. Hollow fiber (Capillary Membrane, polypropylene) with an inner diameter of 330 µm, wall thickness of 150 µm, and max pore size 0,47µm was found to perform the best and the most reproducible extraction of the analytes. Zolpidem, zolpidem-d6, midazolam, midazolam –d5 and 1-hydroxy midazolam were sufficiently extracted for quantification. Zopiclone, zopiclone- d8, N-desmethyl zopiclone, clonazepam, 7- aminoclonazepam, alprazolam, 1-hydroxy alprazoalm, zopiclone, zopiclone-d8, zopiclone-N-oxide and N-desmetyl zopiclone hydrochloride were not extracted using HF-LPME as extraction method.
The optimized method of HF-LPME was suited for the extraction and pre-concentration of some basic benzodiazepines in sewage water. 1- hydroxy midazolam and midazolam were detectable and zolpidem was found quantifiable in samples taken in January. In April neither of the analytes was quantifiable but they were all detectable.

Extraction with non synthetic organic solvents like plant oils as organic phase is preferable. Synthetic organic solvents like 1- octanol and dihexyl ether are relative expensive and provides a higher health risk of the laboratory personnel and environment compared to plant oils and other non synthetic organic solvents. The tested plant oils and essential oils performed the extraction, but because dihexyl ether provided better extraction results it was preferred as organic phase.

HF-LPME may not be an optimal extraction method for all analytes. Easily hydrolysed groups within the analytes, which may undergo basic or acidic hydrolysis, should be considered before extraction.

12 FUTURE PERSPECTIVES

In order to asses the consequences of pharmaceuticals in the environment more biological testing of effects should be performed. All pharmaceuticals are constructed to possess reactive groups known to interact with specificity in the body. But the knowledge on their toxic effect on biological systems in the environment needs more attention.

Some of the studied benzodiazepines; clonazepam, 7- aminoclonazepam, alprazolam, 1-hydroxy alprazoalm, zopiclone, zopiclone-d8, zopiclone- N-oxide and N-desmetyl zopiclone hydrochloride were not extracted using the developed HF-LPME method.
An extraction method that previously has been found to perform extraction of these analytes is solid phase extraction (SPE). An extraction method for zopiclone and its metabolites from human plasma have been demonstrated by Mistri et al.[32]. In order to detect zopiclone in environment environmental samples it is suspected that SPE is a better extraction and pre-concentration method. Future analysis of benzodiazepines in environmental samples should include several extraction methods.

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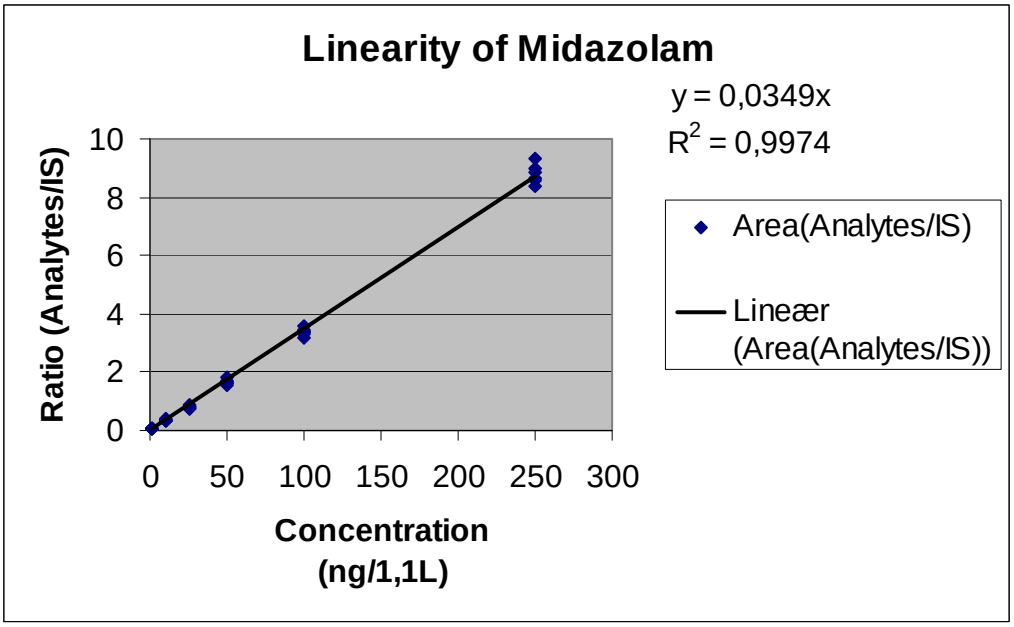
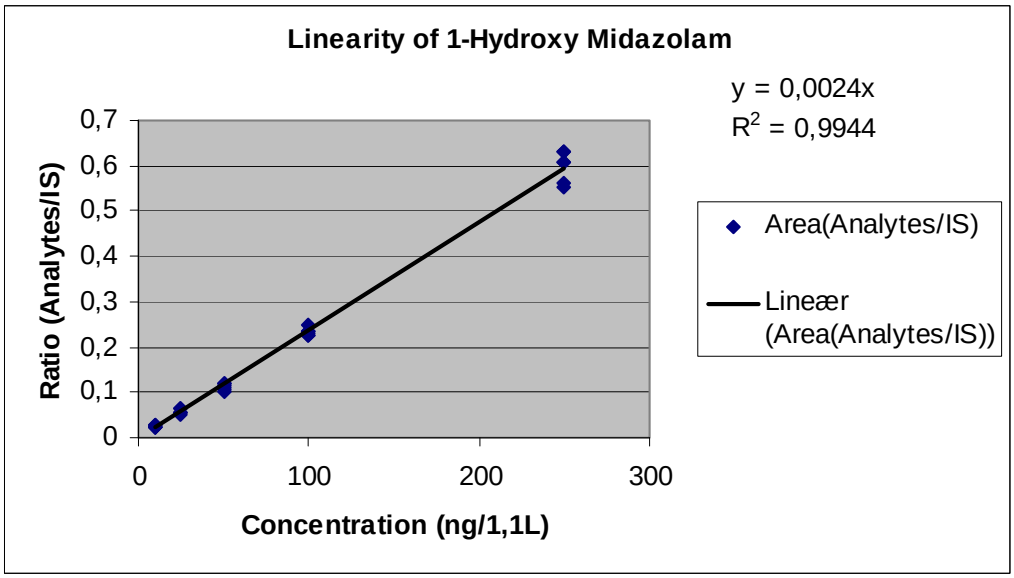
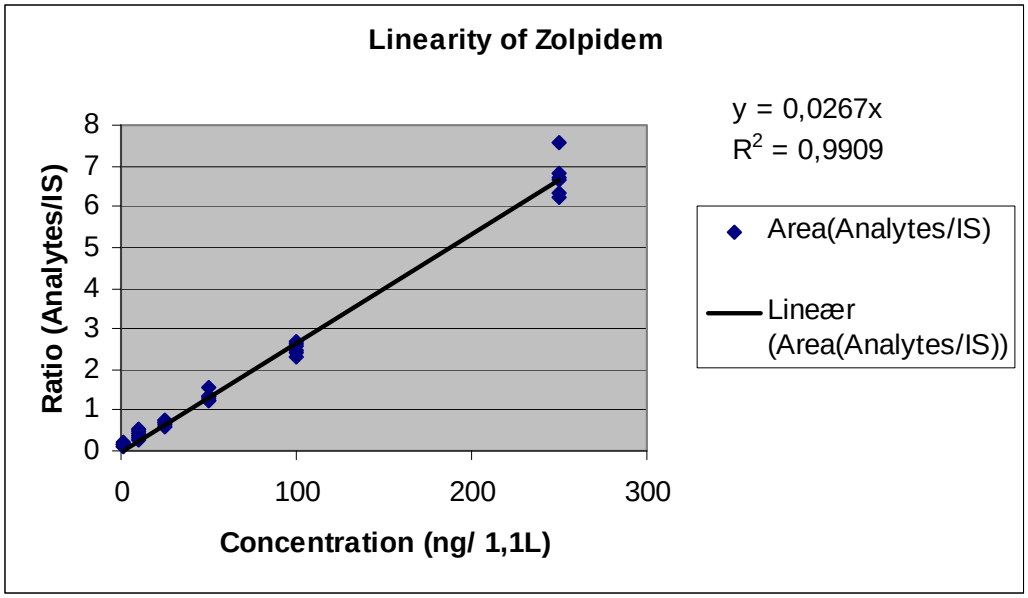
14 APPENDIX

14.1 Linearity of Standards

Linearity of standards for metabolites extracted using LPME







14.2 Multiple reaction monitoring parameters

Dwell time: 0,1 sec.(internal standards) – 0,5 sec. (analytes).

Inter channel delay: 0,01 sec.

Inter scan delay: 0,02 sec.

Repeats: 1

Span: 0,05 amu

14.3 Average area after extraction

Table 7: Different organic phases tested. Water adjusted to 12.65 with sodium hydroxide (pH 12.65) was used as donor phase and water adjusted to pH 2 with formic acid as acceptor phase

Concentration analytes added	Analytes	Sunflower oil	Corn oil	Sesame oil	Rape (seed) oil	Peanut oil	Almond oil	Dihxyl ether	Peppermint oil	Eucalyptus oil	1- Octanol	3- Octanol
50 ng	**1-Hydroxy Midazolam**	121	209	187	136	155	144	417	-	-	-	-
	Midazolam-d 5	288	414	278	228	415	266	1480	-	-	-	-
	Midazolam	1293	2176	1480	1309	1651	1241	5770	-	-	-	-
	Zolpidem	197	514	387	304	240	279	1224	-	-	-	-
	Zolpidem- d6	341	803	565	467	256	392	1912	-	-	-	-
100 ng	**1- Hydroxy Midazolam**	352	372	366	260	351	219	1250	-	-	-	-
	Midazolam- d5	820	902	725	465	866	426	4481	-	-	-	-
	Midazolam	3691	4267	3807	2708	3558	2461	15440	-	-	-	-
	Zolpidem	643	970	788	487	595	335	25440	-	-	-	-
	Zolpidem- d6	1040	1439	1223	798	939	619	3689	-	-	-	-
250 ng	**1- Hydroxy Midazolam**	787	1024	803	715	943	576	2312	-	-	-	-
	Midazolam- d5	1403	1949	1469	1313	2322	1501	9495	-	-	-	-
	Midazolam	11035	10923	6977	7188	9642	7166	30375	-	-	-	-
	Zolpidem	1968	3243	1722	1329	1731	1118	6975	-	-	-	-
	Zolpidem- d6	3086	5112	2808	2074	2526	1730	9109	-	-	-	-

Table 8: Different donor phases tested. Water adjusted to pH 2 with formic acid was used as acceptor phase and dihexyl ether was used as organic phase.

		Average area after extraction		
Concentration analytes added	**Analytes**	**Water adjusted to pH 12.65 with sodium hydroxide**	**Water adjusted to pH 13.95 with sodium hydroxide**	**Water adjusted to pH 11 with ammonia**
50 ng	**1-Hydroxy Midazolam**	**417**	**134**	**-**
	Midazolam-d5	**1480**	**976**	**-**
	Midazolam	**5770**	**5583**	**-**
	Zolpidem	**1224**	**8946**	**-**
	Zolpidem- d6	**1912**	**16583**	**-**
100 ng	**1-Hydroxy Midazolam**	**1250**	**411**	**-**
	Midazolam- d5	**4481**	**4054**	**-**
	Midazolam	**15440**	**19795**	**-**
	Zolpidem	**2558**	**19114**	**-**
	Zolpidem- d6	**3689**	**33282**	**-**
250 ng	**1-Hydroxy Midazolam**	**2312**	**1139**	**-**
	Midazolam- d5	**9495**	**10823**	**-**
	Midazolam	**30375**	**47764**	**-**
	Zolpidem	**6975**	**48584**	**-**
	Zolpidem- d6	**9109**	**90056**	**-**

Table 9: Different acceptor phases tested. Water adjusted to pH 12.65 with sodium hydroxide was used as donor phase and dihexyl ether as organic phase

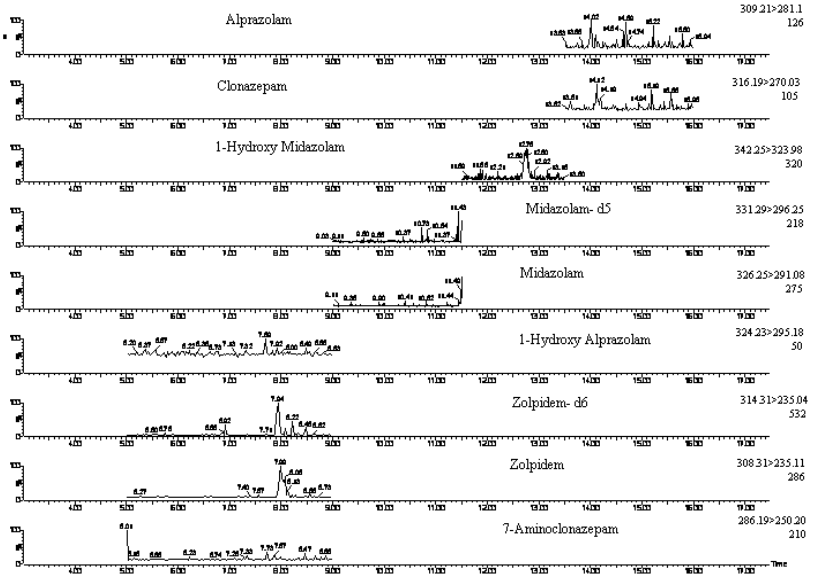
		Average area after extraction		
Concentration analytes added	**Analytes**	**Water adjusted to pH 2 with formic acid**	**Water adjusted to pH 1 with hydrochloric acid**	**Water adjusted to pH 2 with hydrochloric acid**
50 ng	**1-Hydroxy Midazolam**	417	432	-
	Midazolam- d5	1480	574	-
	Midazolam	5770	2415	-
	Zolpidem	1224	1228	-
	Zolpidem- d6	1912	1593	-
100 ng	**1-Hydroxy Midazolam**	1250	851	-
	Midazolam- d5	4481	1050	-
	Midazolam	15440	4348	-
	Zolpidem	2558	2521	-
	Zolpidem- d6	3689	3501	-
250 ng	**1-Hydroxy Midazolam**	2312	2371	-
	Midazolam- d5	9495	3159	-
	Midazolam	30375	12609	-
	Zolpidem	6975	5761	-
	Zolpidem- d6	9109	7620	-

Table 10: Different hollow fibers tested. Water adjusted to pH 12.65 with sodium hydroxide was used as donor phase, dihexyl ether as organic phase and water adjusted to pH 2 with formic acid was used as acceptor phase.

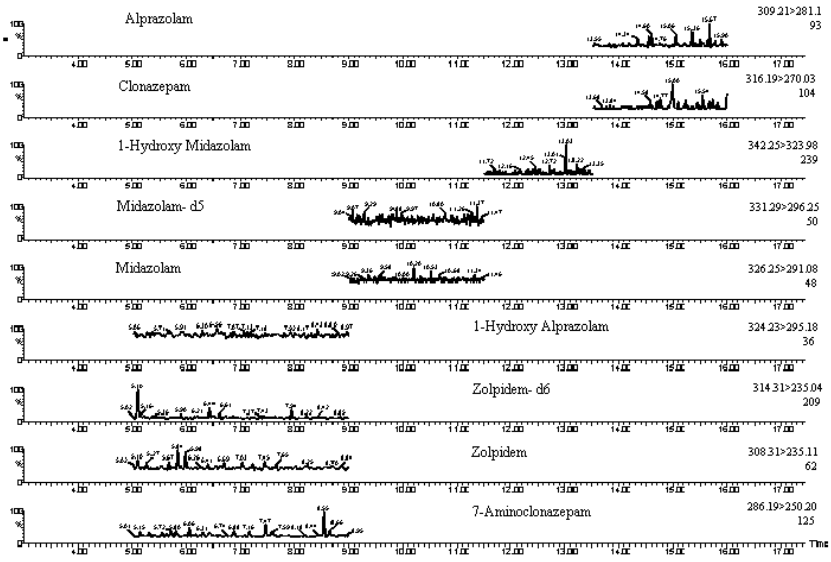
		Average area after extraction		
Concentration analytes added	**Analytes**	**P1 LX**	**PP Q3/1**	**PP Q3/2**
50 ng	1-Hydroxy Midazolam	417	901	1029
	Midazolam- d5	1480	1287	1453
	Midazolam	5770	5400	6185
	Zolpidem	1224	71	179
	Zolpidem- d6	1912	101	255

14.4 Chromatograms of different organic phases, donor phases, acceptor phases and fibers tested during method optimization

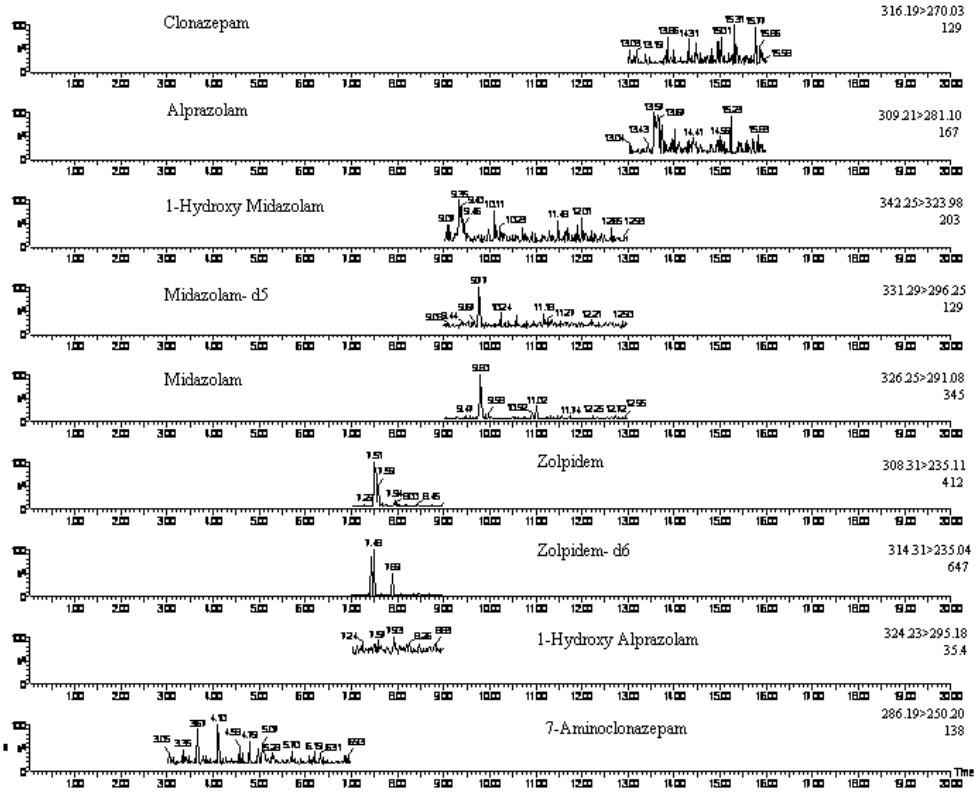
Different organic phases tested



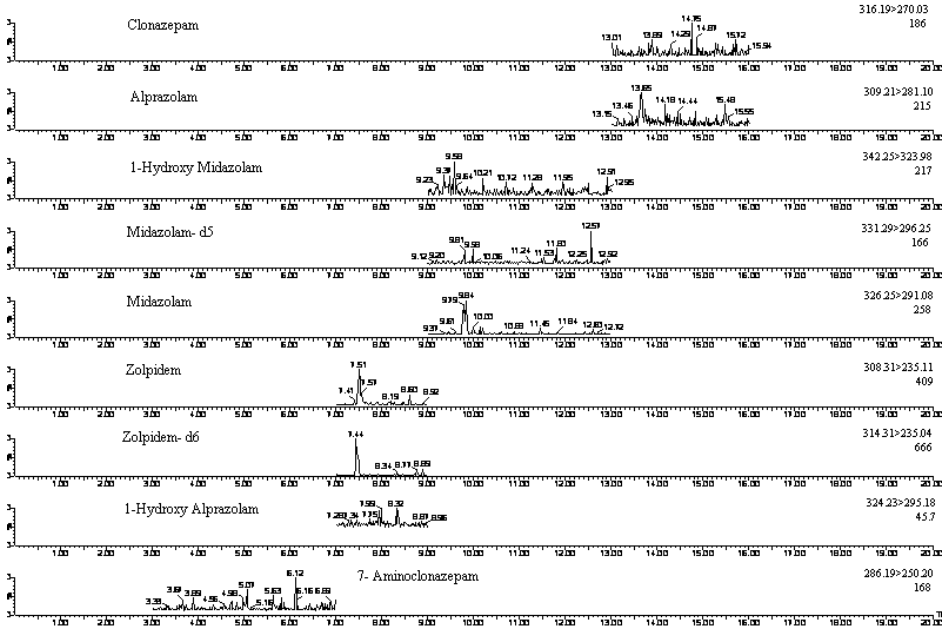
Chromatogram 10: MRM chromatogram. 1-octanol used as organic phase. 100 ng analytes added the donor phase.



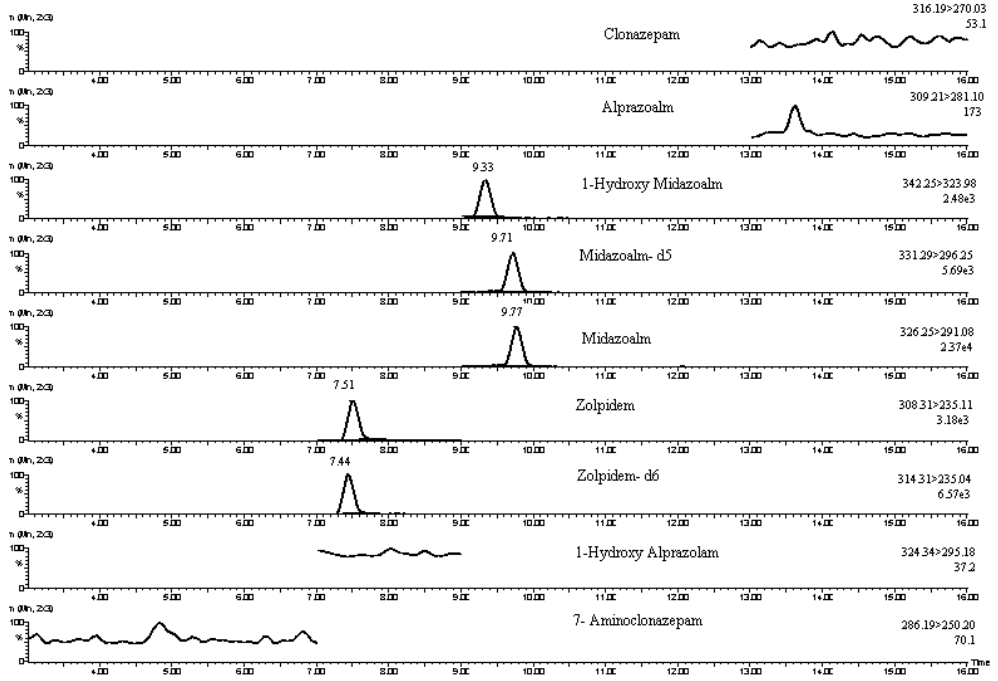
Chromatogram 11: MRM chromatogram. 3-octanol used as organic phase. 100 ng analytes added the donor phase.



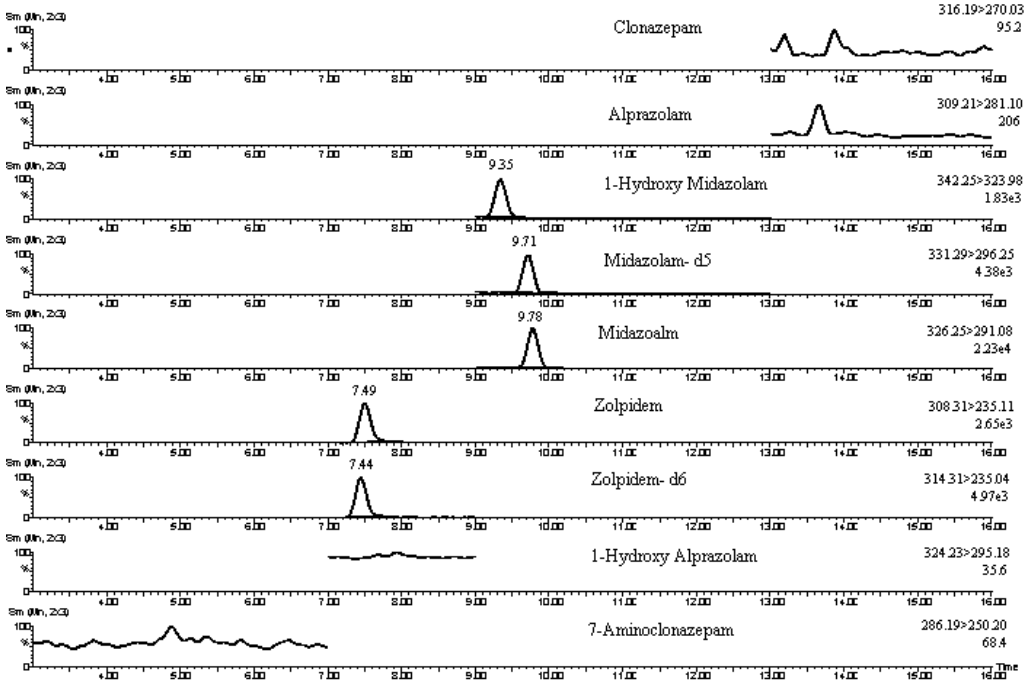
Chromatogram 12: MRM chromatogram. Peppermint oil used as organic phase. 100 ng analytes added the donor phase.



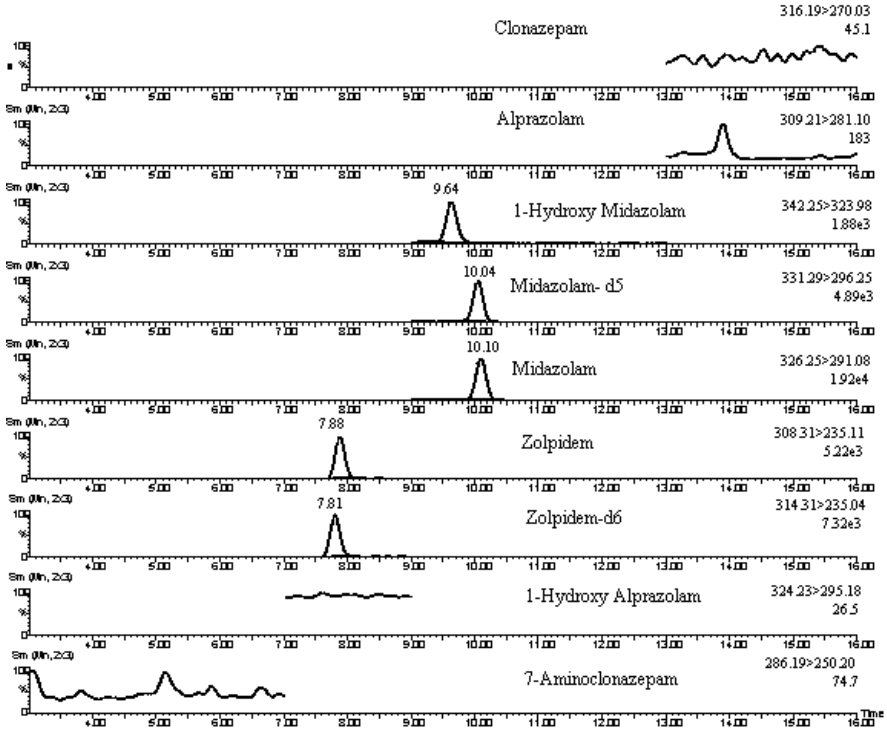
Chromatogram 13: MRM chromatogram. Eucalyptus oil used as organic phase. 100 ng analytes added the donor phase.



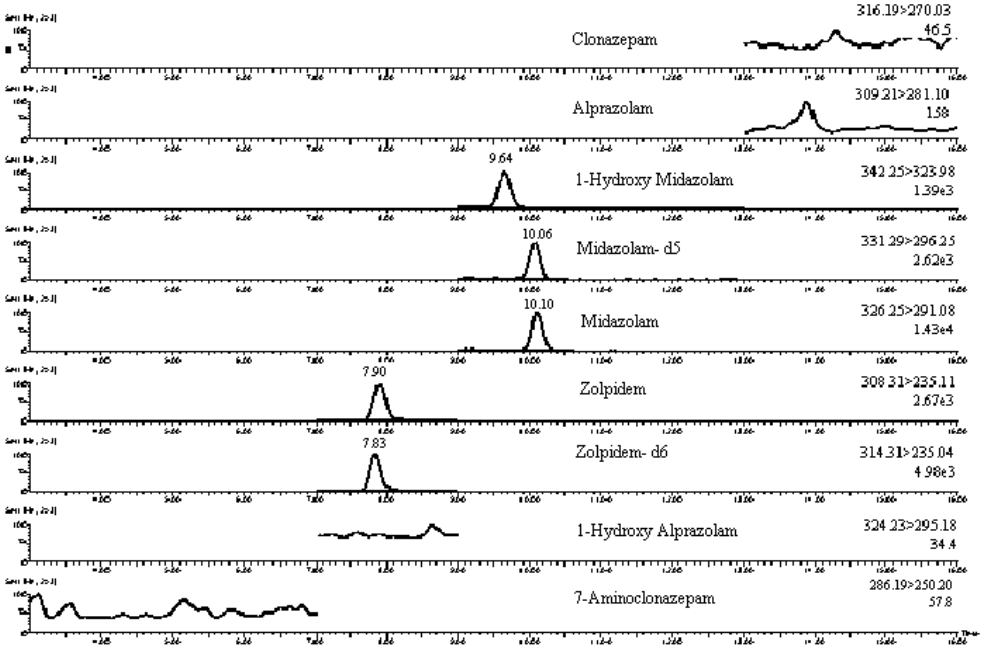
Chromatogram 14: MRM chromatogram after smoothing. Peanut oil used as organic phase. 100 ng analytes added the donorphase.



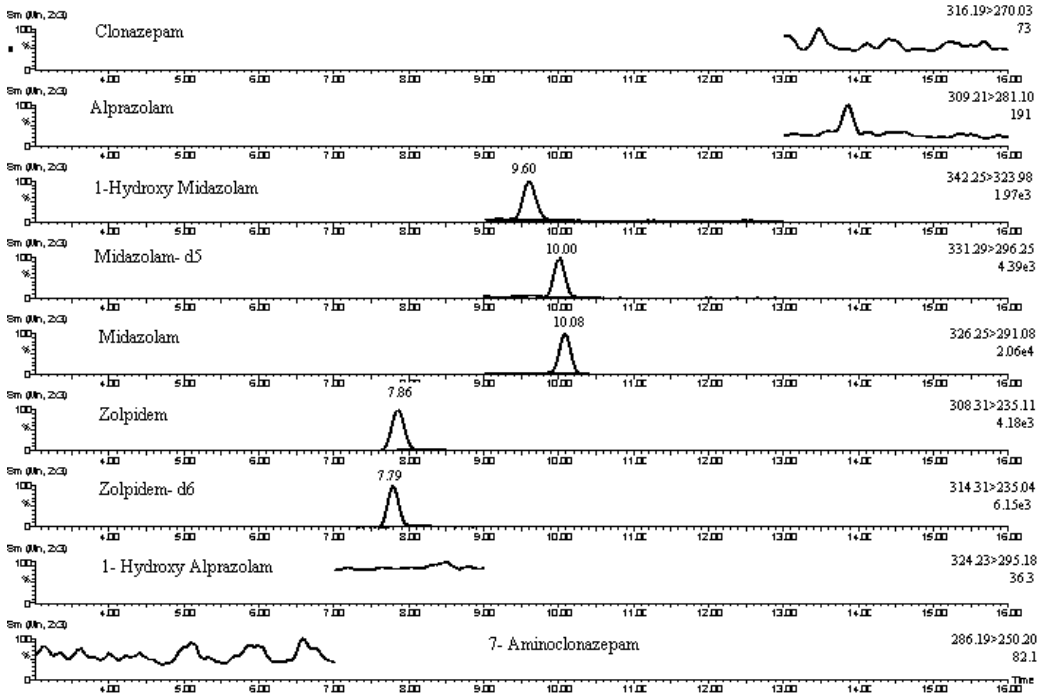
Chromatogram 15: MRM chromatogram after smoothing. Almond oil used as organic phase. 100 ng analytes added the donor pahse.



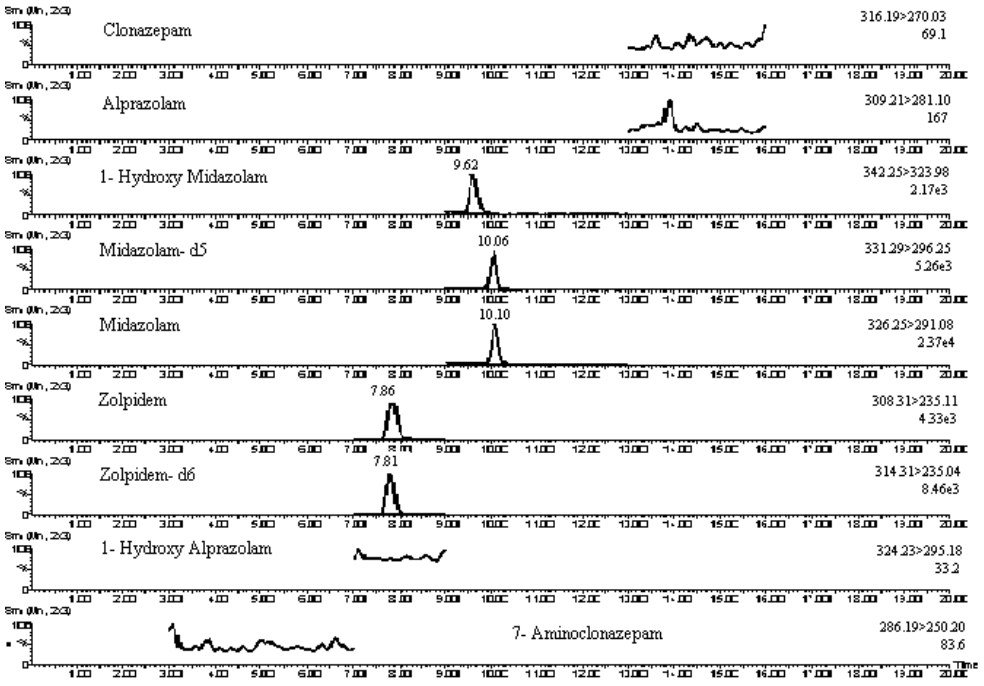
Chromatogram 16: MRM chromatogram after smoothing. Corn oil used as organic phase. 100 ng analytes added the donor phase.



Chromatogram 17: MRM chromatogram after smoothing. Rape (seed) oil used as organic phase. 100 ng analytes added the donor phase.

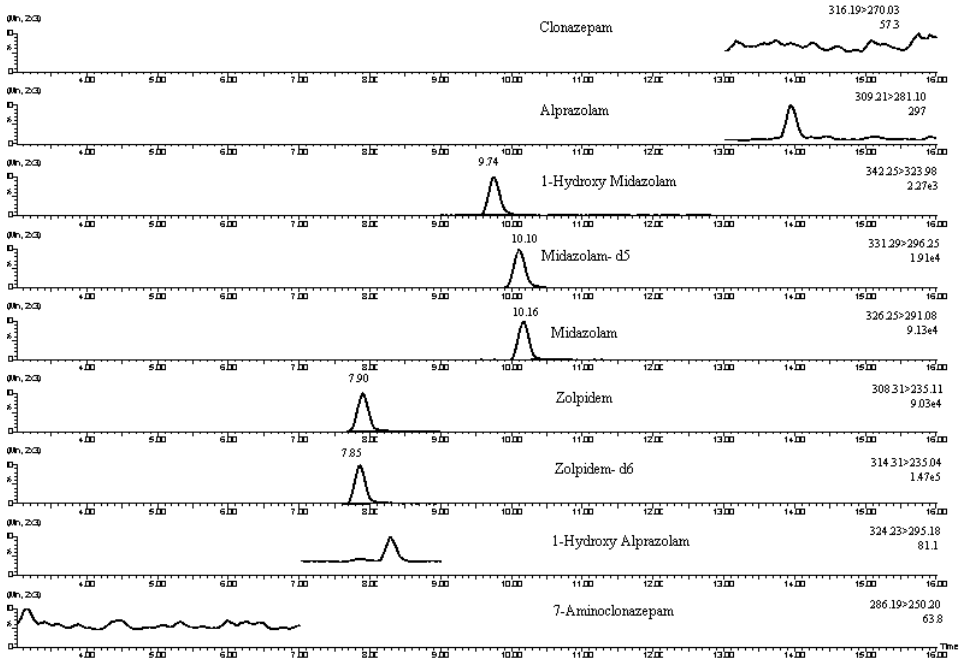


Chromatogram 18: MRM chromatogram after smoothing. Sesame oil used as organic phase. 100 ng analytes added the donor phase.

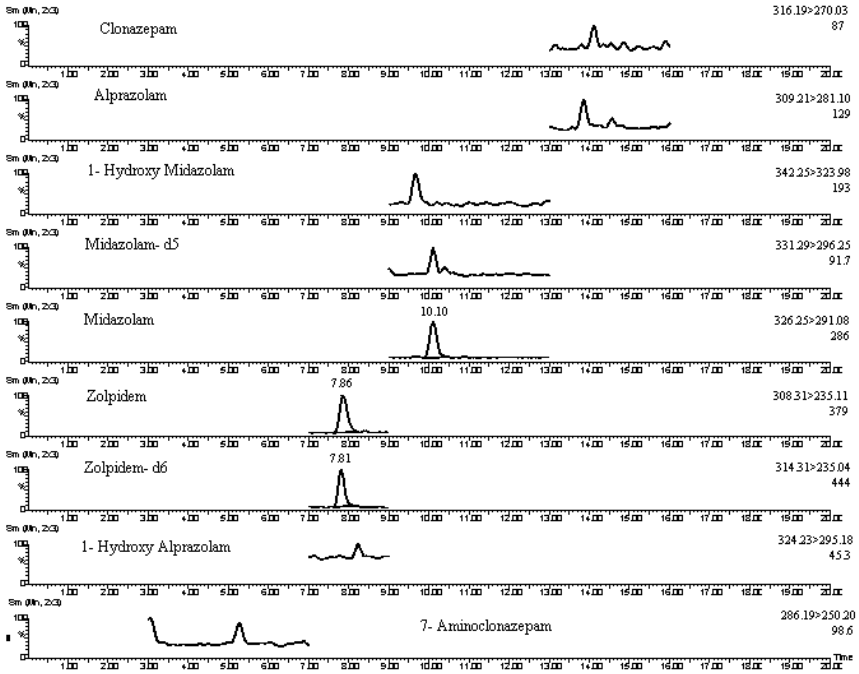


Chromatogram 19: MRM chromatogram after smoothing. Sunflower oil used as organic phase. 100 ng analytes added the donor phase.

Different donor phases tested

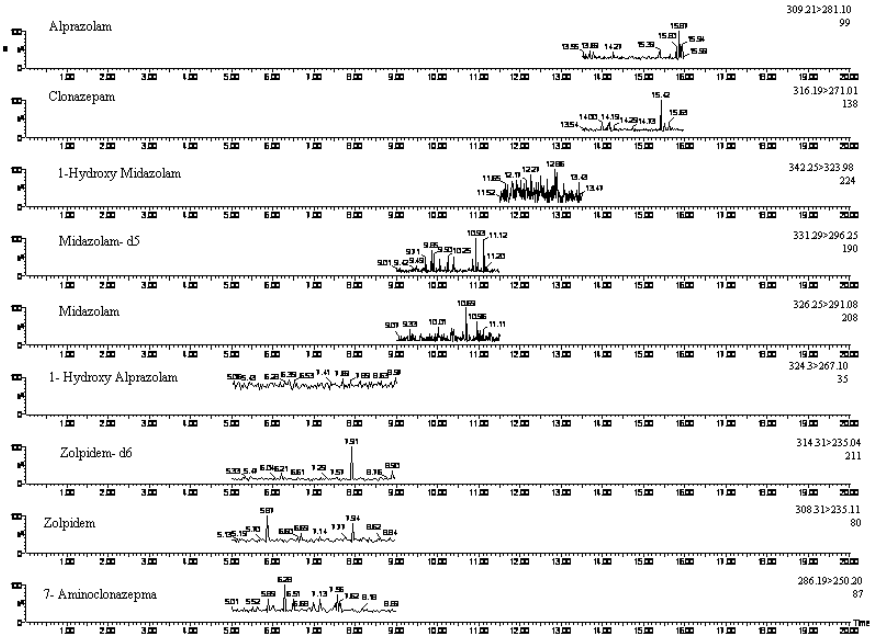


Chromatogram 20: MRM chromatogram after smoothing. Water adjusted to pH 13.95 with sodium hydroxide and added 100 ng analytes used as donor phase.

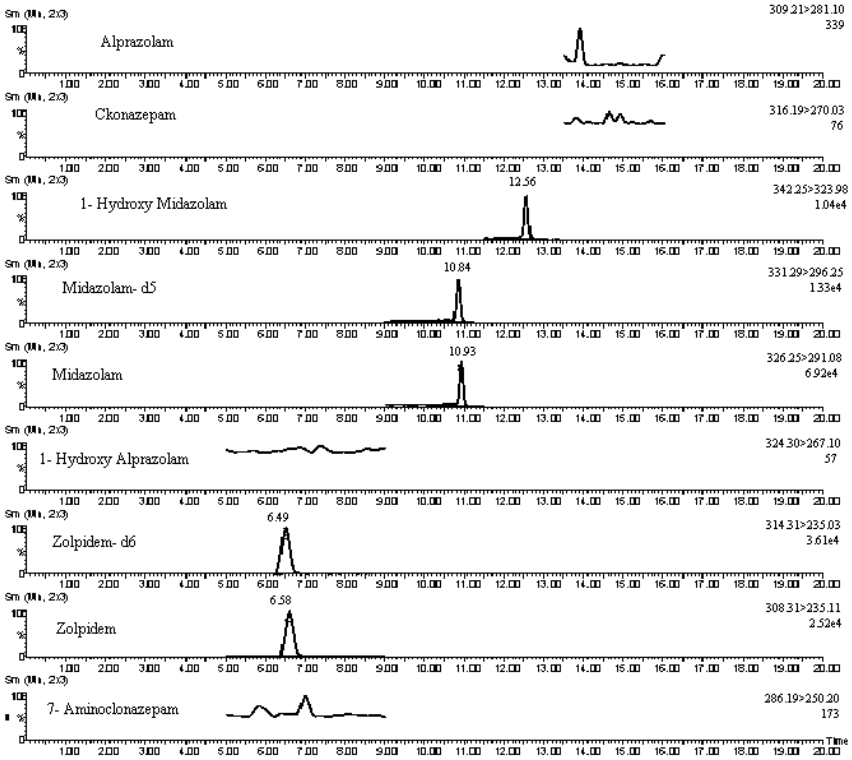


Chromatogram 21: MRM chromatogram after smoothing. Water adjusted to pH 11 with ammonia and added 100 ng analytes used as donor phase.

Different acceptor phases tested

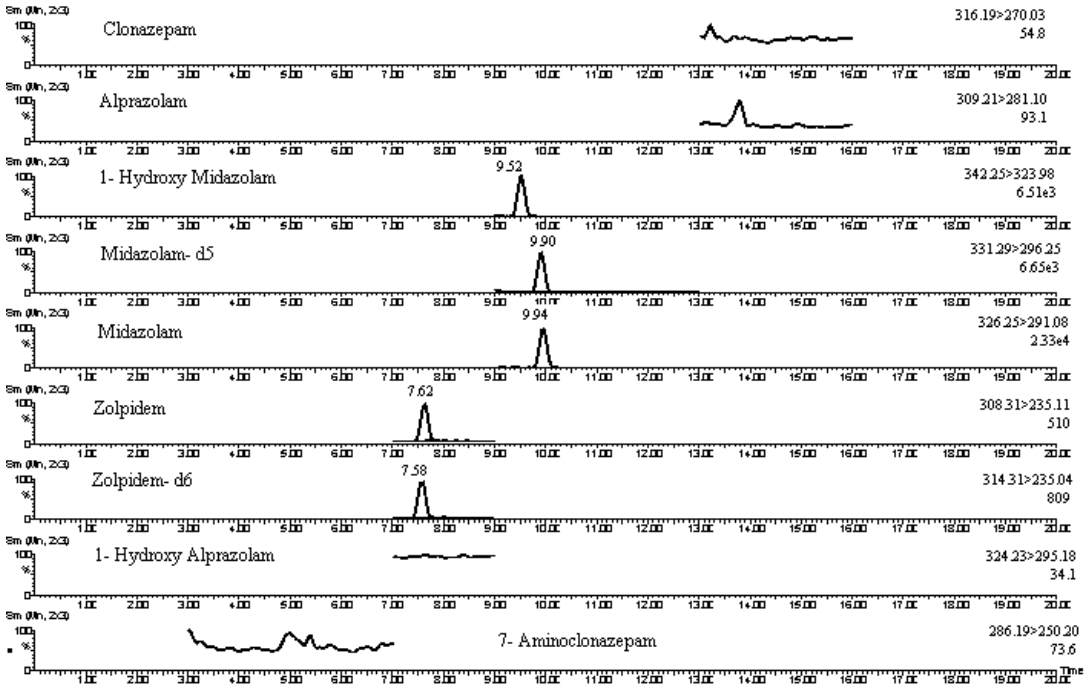


Chromatogram 22: MRM chromatogram. Water adjusted to pH 2 with hydrochloric acid used as acceptor phase. 100 ng added the donor phase



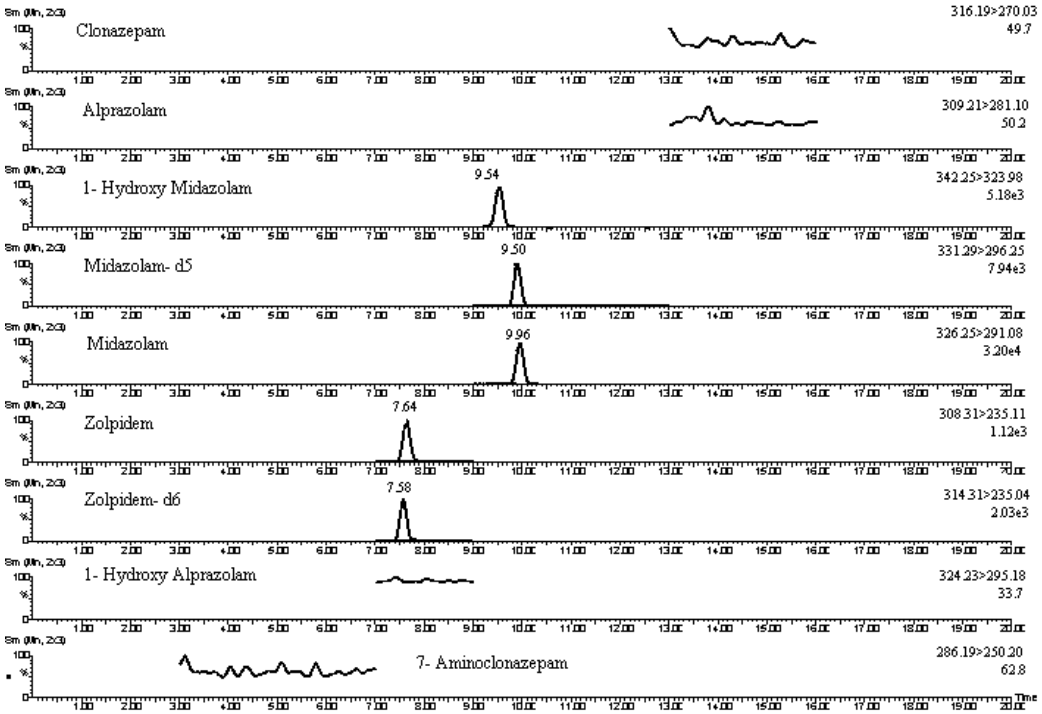
Chromatogram 23: MRM chromatogram after smoothing. Water adjusted to pH 1 with hydrochloric acid used as acceptor phase. 100 ng analytes added the donor phase.

Different fibers tested



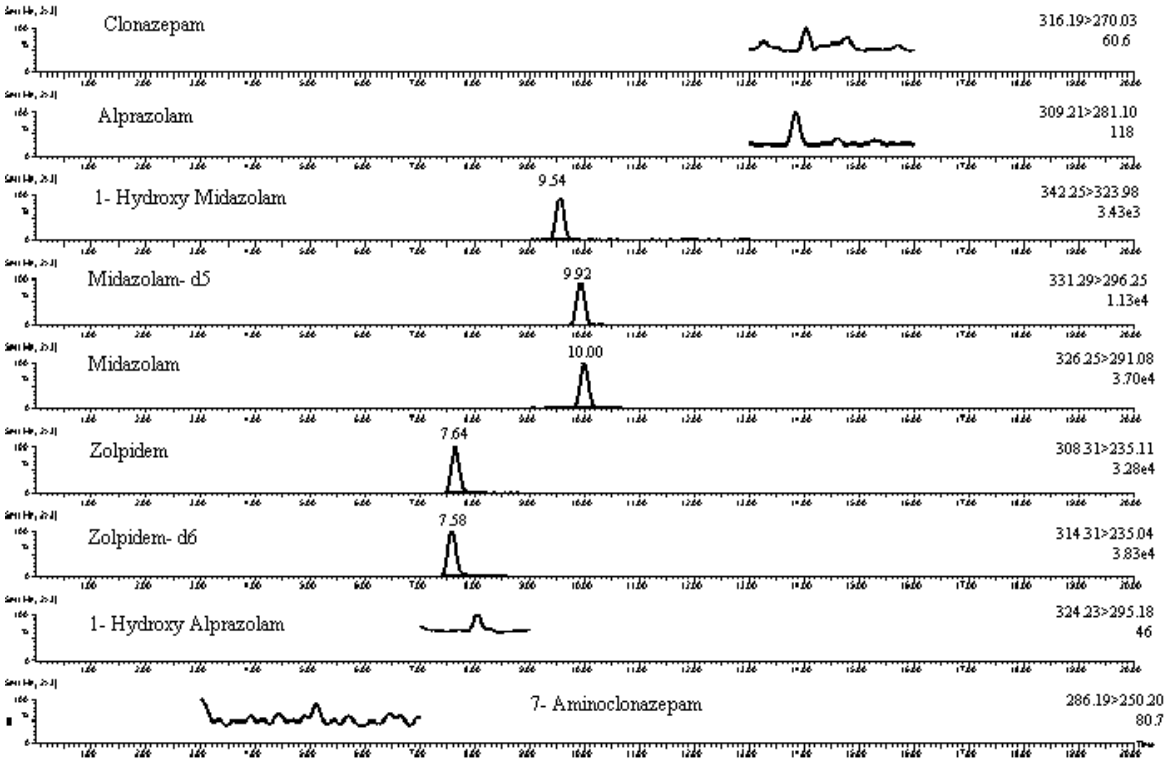
Chromatogram 24: MRM chromatogram after smoothing.

Fiber PP Q3/1 tested. Water adjusted to pH 12.65 with sodium hydroxide was used as donor phase, dihexyl ether as organic phase and water adjusted to pH 2 with formic adic was used as acceptor phase. 50 ng analytes were added the donor phase.



Chromatogram 25: MRM chromatogram after smoothing.

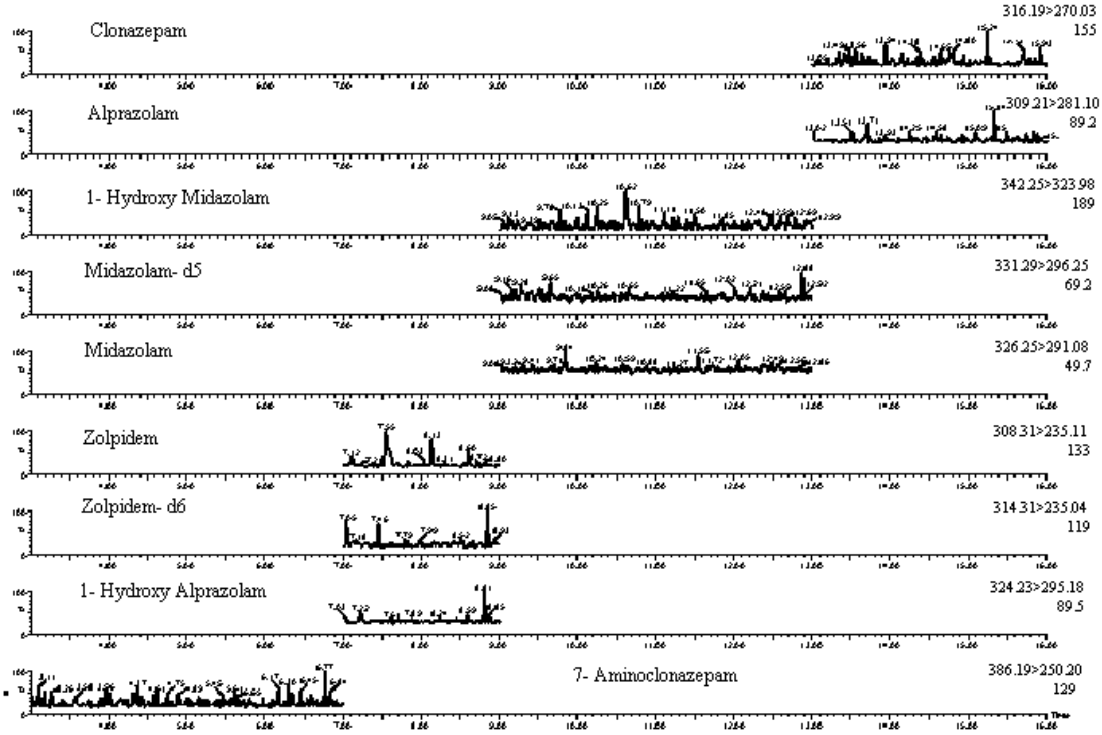
Fiber PP Q3/2 tested. Water adjusted to pH 12.65 with sodium hydroxide was used as donor phase, dihexyl ether as organic phase and formic adic (pH 2) was used as acceptor phase. 50 ng analytes were added the donor phase.



Chromatogram 26: MRM chromatogram after smoothing.

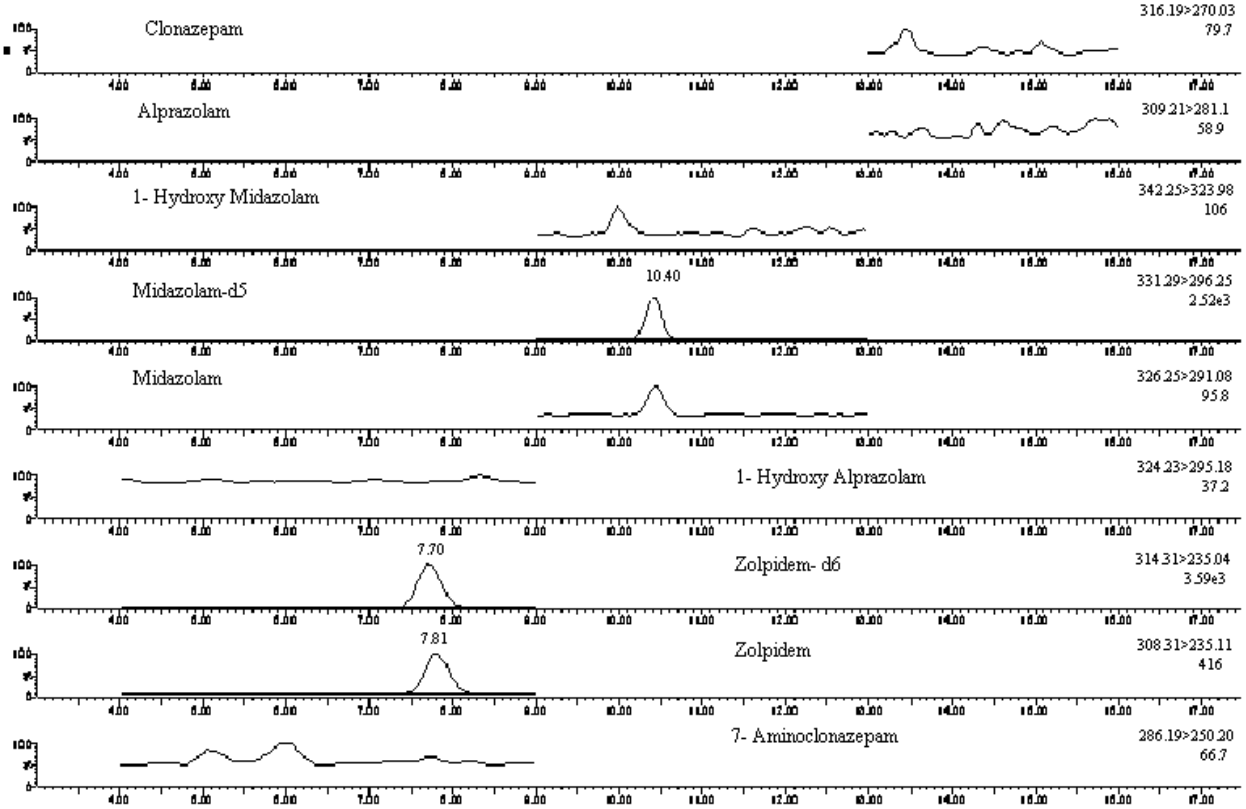
Fiber P1 LX tested. Water adjusted to pH 12.65 with sodium hydroxide was used as donor phase, dihexyl ether as organic phase and water adjusted to pH 2 with formic adic was used as acceptor phase. 50 ng analytes were added the donor phase.

14.5 Extraction of a blank sample



Chromatogram 27: MRM chromatogram of blank sample.

14.6 **Extracted sewage water**



Chromatogram 28 : MRM chromatogram after smoothing. Waste water added a given concentration internal standards (25 ng zolpidem- d6 and 100 ng midazolam- d5). Sample taken in January.