

**University of South Bohemia in  
České Budějovice  
Faculty of science**

**Occurrence of organic pollutants in constructed wetlands**

Bachelor thesis

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České Budějovice 2013

Bachelor thesis:

Trsková E., 2013: Occurrence of organic pollutants in constructed wetlands, Bc. Thesis, in English – 43 p., Global change research centre AS CR, The University of South Bohemia, České Budějovice, Czech republic

## **Abstract**

Constructed wetlands are wetlands designed to improve the quality of water. In this work, four representatives of typical organic pollutants in Constructed wetlands are studied – DEET, cotinine, coprostanol and galaxolide as the representatives of insecticide, alkaloid, faecal sterol and musk compound respectively. Moreover three different types of extraction techniques – aqueous two phase extraction (ATPE), liquid-liquid extraction (LLE) and stir bar sorptive extraction (SBSE) - are investigated with respect to the overall extraction yield regarding the above mentioned compounds.

## **Abstrakt**

Umělé mokřady jsou navrženy tak, aby zlepšovaly kvalitu vody. V této práci jsou studovány čtyři zástupci typických organických znečišťujících látek nacházejících se v umělých mokřadech – DEET, kotinin, koprostanol a galaxolid jako zástupci insekticidů, alkaloidů, fekálních sterolů a pižma. Dále jsou porovnávány tři různé typy extrakčních technik – vodná dvoufázové extrakce (ATPE), kapalinová extrakce (LLE) a extrakce tuhým sorbentem na míchací tyčince (SBSE) - na základě výtěžku extrakce výše zmíněných sloučenin.

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## **Acknowledgement**

I would like to thank to Prof. Ing. Jan Tříska, CSc. for supervising of my theses and for answering all of my tiring emails.

My big thanks go also to Ing. Martin Moos, PhD. for helping me during work, explaining all of the calculation and for getting up early every day to be in laboratory with me.

To all members of the Global change research centre AS CR I would like to thank for creating such a nice and friendly atmosphere.

Last but not least I would like to thank to my family, boyfriend and friends for being patient and supportive all the time, especially when writting the bachelor thesis.

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## List of abbreviation

AHTN = 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline  
ATPS = Aqueous two phase systems  
ATPE = aqueous two phase extraction  
BOD = Biochemical Oxygen Demand  
BSTFA = N,O-Bis(trimethylsilyl)trifluoro-acetamide  
COD = Chemical Oxygen Demand  
CW = Constructed wetlands  
DCM = dichloromethane  
DEET = N,N-diethyl-3-methylbenzamide  
GC = Gas chromatography  
GC-MS = Gas chromatography – Mass spectrometry  
HHCB = Galaxolide  
LLE = liquid-liquid extraction  
LOD = Limit of detection  
LOQ = Limit of quantification  
ND = Not detected  
OTNE = 1-(1,2,3,4,5,6,7,8 Octahydro-2,3,8,8-tetramethyl-2-naphthalenyl) ethanone  
PDMS = Polydimethyl siloxane  
PTV = Programmed temperature vaporization  
RDX = cyclotrimethylenetrinitramine  
SBSE = stir bar sorptive extraction  
TMS = trimethylsilyl  
TNT = Trinitrotoluene  
WWTP = waste water treatment plants

# 1 Introduction

The treatment of waste water is a very important topic nowadays. Researchers are trying to find the most ecological and economical way to get rid of potentially hazardous substances. One of the best possibilities beside large waste water treatment plants (WWTP) is to use constructed wetlands. In historical times, wetlands were considered undesirable and useless areas. However research, done in 20<sup>th</sup> century changed the general opinion of them. Not only do they constitute natural flood control barrier, but they are also able to act as water treatment systems to maintain the stability of ecosystems. Both of these advantages are made use of in constructed wetlands.

The stability of ecosystems is crucial for maintaining the stability of the whole environment. The Ramsar convention on wetlands is the intergovernmental treaty that provides the scope for the international cooperation which leads to conservation and wise use of wetlands and their resources. The Convention covers a broad definition of the types of wetlands, including lakes, rivers, swamps and marshes, wet grasslands, oases, deltas, mangroves and coral reefs and also the human-made places, such as fish ponds, rice paddies, reservoirs, and salt pans. The main goal of the Ramsar convention is to maintain the ecological character of wetlands and to sustainably use the wetlands and their resources for the benefit of humankind (<http://www.ramsar.org>).

The beginning of constructed wetlands is considered to be in the early fifties of the 20th century, when Käthe Seidel conducted the first experiment of using macrophytes for wastewater treatment in Germany. Afterwards a number of different experiments using constructed wetlands to degrade various pollutants in wastewater were conducted (Haberl et al., 1995). At first the constructed wetlands were only used for the treatment of heavy metals, nutrients, phosphorus and nitrogen, but recent research has shown that they also possess a high capacity for the treatment of different organic pollutants and till the beginning of 21st century, the technology of constructed wetlands was used to treat more than 20 different types of wastewater in Europe (Vymazal, 2011).

Constructed wetlands are wetlands that have been designed to improve the quality of water. The same biological and chemical processes take place in them as in natural wetlands, but they have been constructed in areas where needed. The main components of constructed wetlands are vegetation and soil, sediment and hydrology as a main process. As recently has been shown, different plant species and soil (sediment) types can be used for treatment of

different organic pollutants. The region of the roots – the rhizosphere - is the most reactive area of constructed wetlands.

The wetlands are nowadays one of the best and cheapest wastewater treatment systems used, although the real chemical transformations in wetlands are something like a chemical “black box” and we usually know only information about the inflow and the outflow loads.

The main goal of the bachelor thesis was to observe the presence of selected chemicals: DEET, cotinine, coprostanol and galaxolide as the representatives of insecticide, alkaloid (metabolite of nicotine as a smokers activity), faecal sterol (indicator of the faecal contamination of the water effluent) and musk compound respectively in the constructed wetland with horizontal subsurface flow in Slavošovice. In addition, the second goal of the thesis was the investigation of three different types of extraction for the preparation of the samples regarding the above mentioned compounds of the different polarity with the respect to the overall yield of the three types of the extraction – aqueous two phase extraction (ATPE), liquid-liquid extraction (LLE) and stir bar sorptive extraction (SBSE). The final goal was to find the condition for the GC-MS analysis of the above mentioned compounds.



## 2 Constructed wetlands

Wetlands are recognized as transitions between dry land and open water – or as intermediate between terrestrial and aquatic ecosystems. The most important components are vegetation and soil, sediment and hydrology. Different soil types and plant species are used in constructed wetlands.

### 2.1 Classification of wetlands

#### 2.1.1 Dominant macrophytes

Wetlands are classified regarding dominant macrophytes according to Brix (1994) into:

**Free-floating macrophyte-based systems:** macrophytes are not rooted and they float freely on or in the water column – usually limited to non turbulent, protected areas. (Haberl, 2003)

**Submerged macrophyte-based systems:** these macrophytes can live at all depths within the photic zone. Vascular angiosperms are able to live in depths up to 10 m deep but non vascular macro-algae live to the lower limit of the photic zone – up to 200 m. (Haberl, 2003)

**Rooted emergent macrophyte-based systems:** macrophytes grow on water saturated or possibly submersed soil with water level about 0.5 m below the soil surface to 1.5 m above the soil surface. (Haberl, 2003)

#### 2.1.2 Water flow

According to water flow we can classify wetlands into:

**Surface flow systems** – are densely vegetated and their typical water depth is less than 0.4 m

**Horizontal/vertical subsurface flow systems** – the technology is based on the work of Seidel (1967) and nowadays is worldwide applied. These wetlands have a bed of soil as a substrate for the growth of macrophytes. Wastewater gravitationally flows either in horizontal or in vertical direction through the bed substrate while contacting a mixture of facultative microbes living in the rhizosphere. The bed depth is usually between 0.6-1.0 m. (Haberl, 2003)

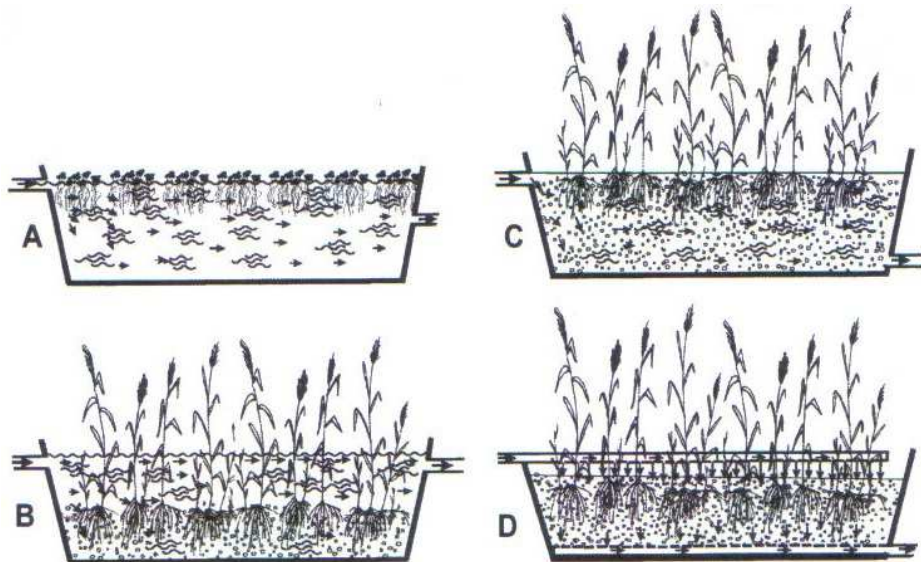


Figure 1: Wetland systems – A, pond with free-floating plants; B, horizontal surface flow wetland; C, horizontal subsurface flow wetland; D, vertical flow wetland (Stottmeister et al., 2003)

## 2.2 Vegetation

The advantage of wetland plants is that they are worldwide similar due to free water supply and very hostile environment which plant roots must survive. Moreover these macrophytes had to find a structural mechanism to avoid root anoxia – by the evolution of air spaces (aerenchyma) in roots and stems. These spaces allow diffusion of oxygen from the parts of the plant above water level into the roots.

## 2.3 Rhizosphere

The region of the roots – the rhizosphere - is the most reactive area of constructed wetlands. According to Hiltner and Störmer (1903) the term rhizosphere can be divided into the endorhizosphere, the root interior, and ectorhizosphere, the root surrounding. The zone where these areas meet is known as the rhizoplane (Elliot et al., 1984) and it is the region of most intensive interaction between plant and micro organisms.

## **2.4 Plant's physiology**

In order to learn more about degradation of organic pollutants in the root zone we need to know something about physiology of plants. The first important process in helophytes is gas transport connected with oxygen release, which is provided by aerenchyma. These gas chambers are gas permeable but provide secure barriers against liquid penetration. The gas flow in them is driven by diffusion – this process enables not only respiration under anoxic conditions but also drives release of oxygen into the rhizosphere, which causes formation of an oxidative protective film. This constant release of oxygen into the rhizosphere is very important for water treatment.

The second important aspect is the interaction between roots and soil matrix. The soil acts as the supporting material for plant growth and microbial films and has great influence on hydraulic processes. Both chemical composition and physical parameters (e.g. grain size) are important factors influencing bio system – by indicating the flow of wastewater in constructed wetlands and ultimately the removal of contaminants. On the one hand, roots and microbial biomass clog up soil pores, but on the other hand, root growth and the microbial degradation of dead roots cause the formation of new pores.

The third process which plays minor role in wastewater treatment is the uptake of inorganic compound by plants. Plants are able to tolerate high concentrations of nutrients and heavy metals and in some cases even to accumulate them in their tissues. Nevertheless the uptake of compounds like nitrogen, phosphorus or heavy metals is too low to have a significant effect on waste water treatment.

Other processes influencing the possibility of water treatment are: the release of carbon compounds from plants, transpiration, and the role of microbial degradation or transformation of organic and inorganic pollutants (especially nitrification – denitrification) and temperature. Moreover according to research performed by Seidel (1971) there is even a bacterial effect of higher plants on pathogenic germs.

## **2.5 Species used in constructed wetlands**

From the practical experience it was found that species of helophytes (marsh plants) work best of all in wastewater treatment system. This is because they are able to survive under extreme conditions including: acidic or alkaline pH, toxic wastewater components,

salinity, etc. The most widely used species for wastewater treatment are common reed (*Phragmites australis spp.*), rushes (*Juncus spp.*), bulrushes (*Scirpus spp.*), narrow – leaved cattail (*Typha angustifolia L.*), broad-leaved cattail (*Typha latifolia L.*), yellow flag (*Iris pseudacorus L.*), sweet flag (*Acorus calamus L.*), reed grass (*Glyceria maxima*) and sedges (*Carex spp.*) (Stottmeister et al., 2003). Moreover different plant species are able to treat different chemical compounds.

## **2.6 Application of constructed wetlands**

In general, constructed wetlands are cost-effective and technically feasible for the wastewater treatment. They are not only less expensive and easy to build, but also their operations and maintenance expenses are lower in comparison with other treating possibilities. One of the biggest advantages of constructed wetlands is that they utilize natural processes only, which are also connected with their ability to tolerate flow fluctuations having high stability (buffering capacity). Moreover they provide habitat for water organisms and they do not disturb the landscape. Due to their environmental sensitivity they are popular in general public.

At first the wetlands were used only for the treatment of phosphorus, nitrogen and heavy metals, but recent research has shown, that they are able to treat organic pollutants from domestic wastewater, agricultural wastewater, food wastes and industrial wastewater. (Langergraber et al., 2003)

## **2.7 Removal of organic pollutants**

Many constructed wetlands treat domestic wastewater. As parameters for organic matter removal both BOD and COD (Biochemical and Chemical Oxygen Demand) are used.

Major mechanisms for the organic compounds removal in the constructed wetlands are volatilization, photochemical oxidation, sedimentation, sorption, microbial degradation, aerobic and anaerobic respiration, bioaugmentation of the sediment and sorption by macrophytes. (Langergraber, 2003)

The biggest problem with treatment of large amounts of organic pollutants as suspended solids is the clogging of the substrate pores, particularly in substrate flow wetlands. For this reason plants with low levels of refractory compounds in their litter are used. (Tanner et al., 1998)

The removal efficiency for organic contaminants is usually quite high. However we do not know the exact pathways of the removal. Further research is needed to understand these mechanisms of the wastewater treatment in the constructed wetlands and keep them as effective as possible.

**Table I:** Plant types used for specific organic pollutions<sup>1</sup>

| Pollutant type  | Plant used   | Reference                     |
|---|--|-------------------------------|
| Hydrocarbons  | <i>Phragmites spp.</i>                               | Lakatos 2000                  |
|   | <i>Phragmites australis</i>                          | Simi 2000                     |
|   | <i>Typhia spp.</i>                                   | Revitt and Omari et al. 2000  |
|   | <i>Scirpus californicus</i>                          | Campagna and Marques 2000     |
| Oil and Grease  | <i>Typhia spp.</i>                                   | Perdomo et al.                |
| Mineral oils  | <i>Phragmites spp.</i>                               | Ji et al. 2002                |
| Chlorinated volatiles   | <i>Typha latifolia</i>                               | Langergraber 2003             |
| Aromatics   | <i>Phragmites spp.</i>                               | Langergraber 2003             |
|   | <i>Rumex hydrolapatum</i>                            | Langergraber 2003             |
|   | <i>Schoenoplectus spp</i> & <i>Salix spp.</i>        | Langergraber 2003             |
| Glycols   | <i>Phragmites spp.</i>                               | Revitt and Worall et al. 2000 |
| Atrazine  | <i>Typha latifolia</i>                               | Runes et al. 2001             |
| TNT   | <i>Heteranthera dubia</i>                            | Best et al. 1999              |
| RDX   | <i>Scirpus cyperinus</i>                             | Best et al. 1999              |
| General organics  | <i>Phragmites australis</i>                          | Pucci et al. 2000             |
|   | <i>Brachiaria arrecta</i> and <i>B.mutica hybrid</i> | Ide et al. 2000               |
|   | <i>Phragmites spp.</i>                               | Herold et al. 2000            |
|   | <i>Monochoria vaginalis Presl.</i>                   | Junsan et al. 2000            |
| caffeine, salicylic acid, methyl dihydrojasmonate, carboxy-ibuprofen, ibuprofen, hydroxy-ibuprofen, | <i>Phragmites australis.</i>                         | Matamoros and Bayona 2006     |

<sup>1</sup> (Langergraber et al., 2003)

|   |   |                              |
|---|---|------------------------------|
| and naproxen  |   |                              |
| simazine, alachlor,<br>chlorpyrifos,<br>pentachlorobenzene,<br>pentachlorophenol,<br>endosulfan, lindane,<br>mecoprop | <i>Phragmites<br/>australis.</i>                          | Matamoros and<br>Bayona 2007 |
| HHCB,<br>AHTN,<br>Triclosan,<br>OTNE  | <i>P. arundinacea,<br/>T. latifolia,<br/>P. australis</i> | Chen et al. 2009             |

## 3 Studied compounds

### 3.1 DEET

N,N-diethyl-3-methylbenzamide (DEET) is a very popular and commonly used insect repellent, used for the protection against biting insects and also for the control of disease transmission. This active ingredient of most commercial available insect repellents is considered safe and effective, however it is suspected from considerable skin absorption. (Karr et al., 2012)

DEET uses the interference with the sensory perception of insects to lactic acid on the skin of its hosts, which is usually the main stimulus used by insects for attraction and location of their hosts. This compound has commonly been detected in water samples around the world, which indicates that DEET is persistent and able to enter the aquatic environment. The major pathway of its entering the aquatic environments is via washing off and absorption/excretion by humans. The data on the ecological toxicity and the ecological risk assessment of DEET are very sparse, but it has been found out that DEET is persistent to breakdown by water (hydrolysis). (Costanzo et al., 2007)

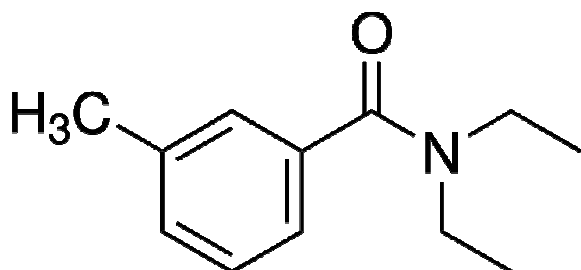


Figure 2: The structure of DEET (<http://wiki.colby.edu>)

### 3.2 Cotinine

Cotinine is the major metabolite of nicotine found in the urine of smokers and for this reason it is widely used as the most common biomarker of nicotine and consequently as the biomarker of the smokers activity. (Parzynski et al., 2008)

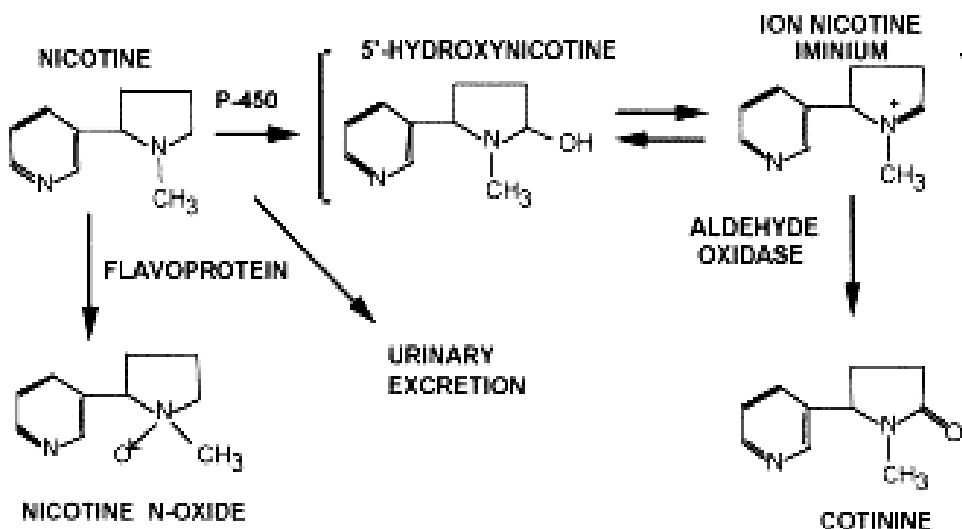


Figure 3: The metabolism of nicotine (Ceppa et al., 2000)

### 3.3 Coprostanol

Coprostanol is a faecal sterol that has been proposed as a possible measure of faecal pollution. (Jeng et al. 1996; Eneroth et al., 1964; Gérard et al., 2005). Coprostanol is produced mainly in the intestines of mammals by the microbial reduction of cholesterol, the main sterol found in the tissues of vertebrates.

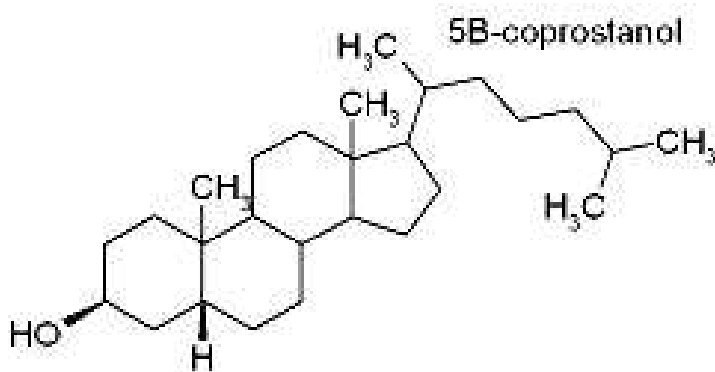


Figure 4: The structure of Coprostanol (<http://www.bgs.ac.uk>)

### 3.4 Galaxolide

Galaxolide (HHCB; 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta(g)-2-benzopyran) is a polycyclic musk, which is commonly used in fragrances, detergents, air



fresheners and perfumes. It is the most widely used polycyclic musk. Its considerable amounts have been detected for example in the sewage effluents (0.2-6.0  $\mu\text{g/L}$ ), sewage sludge and the fresh water at Magdeburg (Winkler et al., 1998). Moreover Galaxolide bioaccumulates in aquatic organisms, which was indicated by the food chain transfer in aquatic ecosystems. (Gatermann et al., 2002)

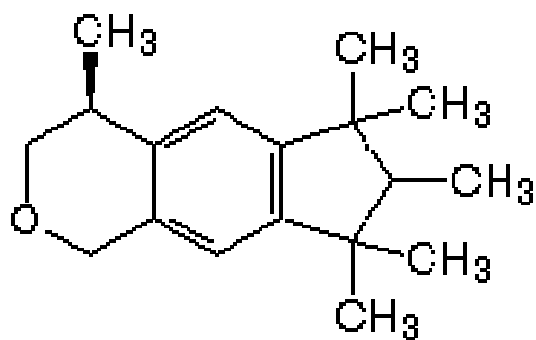


Figure 5: The structure of Galaxolide (<http://www.chm.bris.ac.uk>)

## **4 Theory of experimental methods**

### **4.1 Extraction techniques:**

#### **4.1.1 Aqueous two-phase extraction (ATPE)**

ATPE is a very promising technique of liquid-liquid extraction used mainly for the purification of biomolecules. It combines high selectivity and biocompatibility and moreover it is relatively easy to perform. (Barros, 2009)

Aqueous two phase systems (ATPS) form spontaneously when the two structurally different components in aqueous solutions are mixed above a certain critical concentration. The two components can be two polymers or a polymer and the salt, a short chain alcohol and the salt etc.

ATPE has been successful in processing of several biological compounds including proteins, amino acids and nucleic acids. (Albertsson 1986). Its advantage is that it can be performed by using the same instrumentation as the traditional liquid-liquid extraction.

However, the complexity of the system and the partition mechanism, which is still poorly understood, makes this technique usually demanding for the application in the wide range of processing chemicals or pharmaceuticals. The parameters affecting the partitioning of biomolecules in the ATPS include surface hydrophobicity, charge and size, system composition, electrostatic and van der Waals forces, hydrogen bonds, hydrophobic interactions and steric effects. These all effects makes the behaviour of each different compound fairly unique, but the advantage is that they can be manipulated and changed in order to get the highest possible yield of the desired compound. (Bensch, M. 2007).

ATPE has been also used e.g. for the extraction of metal ions (Bulgariu et al., 2008; Silva et al., 2011), gallic acid (Freire et al., 2012) or gold (Bulgariu et al., 2011).

#### 4.1.2 Stir Bar Sorptive Extraction (SBSE)

Stir bar sorptive extraction was first introduced in 1999 by Baltussen et al. as the sample preparation technique. This technique uses a stir bar coated with a polydimethyl siloxane (PDMS) layer, which has specific characteristics that lead to the great results. The first advantage is that the analytes are not retained on an active surface (which is the case with adsorbents) but they are sorbed into the PDMS phase and they are retained within the bulk of the sorbent. As the sorption is a weaker process than adsorption, there is much lower occurrence degradation of unstable analytes. The second advantage is that compounds can be desorbed at lower temperatures which again leads to lower degradation of thermo-sensitive substances. The third great aspect of SBSE is the fact that the retaining capacity of PDMS for a certain compound is not influenced by the presence of high amounts of water or other analytes because all the solutes have their unique partitioning equilibrium with the PDMS phase. Moreover the degradation fragments of the sorbent contain characteristic silicon mass fragments so they can be easily recognized using the mass selective detector and from that reason they do not interfere with the analysis of unknown samples. (Baltussen et al., 1999)

The Method of SBSE coupled to thermodesorption–gas chromatography–mass spectrometry has been used to investigate the insecticides in water samples. The extraction efficiencies were found to be between 29% and 80 % between different insecticides and the value of efficiency for DEET was 44%. From these results it is obvious that the SBSE is a very comprehensive analysis of insect repellents in the environment. (Rodil et al., 2008)

Moreover the method of SBSE coupled to thermodesorption–gas chromatography–mass spectrometry was successfully developed for determining the synthetic musks in water samples. The method is able to efficiently trap and desorb apolar and semi volatile musks with the limits of quantitation at low  $\text{ng L}^{-1}$  levels. The most abundant musk in wastewater and river water was according to the study galaxolide (HHCB) with the amount of 476-2069  $\text{ng L}^{-1}$  in the urban WWTP influent, which collects wastewater from ca. 120 000 inhabitants, and with the amount of 233-1432  $\text{ng L}^{-1}$  in the urban WWTP effluent. (Marcé., 2011)

### **4.1.3 Liquid-Liquid extraction (LLE)**

Liquid-liquid extraction is a separation process based on the different partitioning of the components to be separated between two liquid phases. The separation is based on the relative solubilities of the components in two different immiscible liquids, usually water and the organic solvent and could be described by distribution constant  $K_D$  (Nernst law). Liquid-liquid extraction is usually applied where direct separation methods such as distillation and crystallization cannot be used, or when the components of the mixture to be separated are heat-sensitive or non volatile. (Müller et al., 2008)

This technique is commonly used, but its need of organic solvent is rather problematic due to their toxicity and costs.

## 5 Experimental

### 5.1 Equipment

**Table II:** The table of used equipment

| Instrument          | Type and manufacturer                       |
|---------------------|---|
| Balances            | Kern EMB 220-I                              |
| Magnetic stirrer    | Ika-C-MAG HS 7                              |
| Magnetic stirrer    | MM2A-150                                    |
| Rotary evaporator   | Laborota 4000 Heidolph                      |
| Analytical balances | R 200D Sartorius research                   |
| Ultrasonic bath     | Ultrasonic compact cleaner UCC 4 Powersonic |

### 5.2 Chemicals

**Table III:** The table of used chemicals

| Compound         | Chemical formula                               | M [g/mol] | Supplier      |
|------------------|--|-----------|---------------|
| Ammonium sulfate | $(\text{NH}_4)_2\text{SO}_4$                   | 132.14    | Sigma-Aldrich |
| Ethanol          | $\text{C}_2\text{H}_5\text{OH}$                | 46.07     | MERCK KGaA    |
| Methanol         | $\text{CH}_3\text{OH}$                         | 32.04     | MERCK KGaA    |
| Toluene          | $\text{C}_6\text{H}_5\text{CH}_3$              | 92.14     | MERCK KGaA    |
| Dichlormethane   | $\text{CH}_2\text{Cl}_2$                       | 84.93     | MERCK KGaA    |
| Hexane           | $\text{C}_6\text{H}_{14}$                      | 86.18     | MERCK KGaA    |
| Cotinine         | $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}$ | 176.22    | Sigma-Aldrich |
| Coprostan-3-ol   | $\text{C}_{27}\text{H}_{48}\text{O}$           | 388.67    | Sigma-Aldrich |
| DEET             | $\text{C}_{12}\text{H}_{17}\text{NO}$          | 191.27    | Fluka         |
| Galaxolide       | $\text{C}_{18}\text{H}_{26}\text{O}$           | 258.41    | LGC-labs      |
| Acetone          | $(\text{CH}_3)_2\text{CO}$                     | 58.08     | Fluka         |

### **5.3 Sampling**

The samples were collected on 18<sup>th</sup> June 2012/12<sup>th</sup> July 2012 in inflow and outflow part of the constructed wetland in Slavošovice (GPS: 48°57'40.781"N 14°39'31.043"E). The water samples were put into the dark 2.5L sampling flasks without the presence of any air bubble. The samples were filtrated at room temperature through the 0.45 µm membrane filter and they were stored in the fridge at 4°C for minimal time.

### **5.4 LLE**

Liquid-liquid extraction (LLE) was performed on 300 mL of sample, which was poured into the separation flasks and let to stay at room temperature for about 2 hours. Afterwards it was mixed with 40 mL of dichloromethane (DCM). The flasks were shaken for 10 minutes with carefully releasing the pressure and then let to stay to separate the phases. The lower phase was filtrated through the funnel stuffed with fibreglass and sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) into the heart shaped flask. The whole extraction was repeated once using 30 mL of DCM instead. The pooled DCM-extracts were evaporated to dryness in the rotary evaporator, which was set to the appropriate temperature and pressure. Finally the flask was rinsed four-times with 1 mL of toluene using ultrasonic bath. The sample was placed in the 4mL vial and put into the freezer. The extraction was performed in triplicate from all the samples.

### **5.5 SBSE**

Stir bar sorptive extraction (SBSE) was performed in 500 mL of sample, which was poured into the Erlenmeyer flask and placed onto the magnetic stirrer. The stir bars were maintained in pure methanol before used. The whole flask was covered with the aluminum foil to avoid any light reactions and let to stir for 6 hours. Afterwards the twister was rinsed in distilled water and the residual water droplets were removed. For liquid desorption of the analytes, the stir bar was put into the 2mL vial together with 1 mL of hexane. The vial was let to stay at room temperature for 1 hour and put into the fridge overnight. After 24 h the stir bar was carefully removed and rinsed with 1 mL of hexane and the vial was put into the

freezer. The twister was put into the methanol to regenerate. The extraction was performed in duplicate from all the samples.

## **5.6 ATPE**

Aqueous two-phase extraction (ATPE) was performed in 50 g (50 mL) of the sample, 20 g of ammonium sulphate and 30 g (38 mL) of ethanol. A predetermined quantity of ammonium sulphate was dissolved in water, and then certain volume of ethanol was added into the ammonium sulphate solution and mixed well to form two phases. After separation of phases the lower phase was discarded and the upper phase was filtrated. The filtrate was placed onto the rotary evaporator, and let to evaporate till completely dry. The flask was then washed with methanol, which was filtrated into the 22mL vial. The solvent was evaporated under a stream of nitrogen at 40°C, the vials were rinsed four-times with 1 mL of methanol using ultrasonic bath to quantitatively transfer the analytes into the 4mL vial which was put into the freezer. The extraction was performed in triplicate from all the samples.

## **5.7 Derivatization**

For derivatization 1 mL of each sample was taken into the 2mL vial and it was evaporated under a stream of nitrogen at 40°C. Afterwards 70 µL of 1% N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 µL of pyridine were added. The mixture was heated at 60°C for 30 minutes and evaporated under a stream of nitrogen at 40°C. The analyte residue was dissolved in 1 mL of hexane.

## **5.8 Standard preparation**

The solutions of galaxolide, cotinine, DEET and coprostanol in concentration of 500 µg mL<sup>-1</sup> were prepared in toluene. After that LLE was performed – one triplet by putting 80 µL of each standard into the 300 mL of distilled water, second triplet was done by mixing 295 mL of distilled water and 5 mL of acetone with 80 µL of each standard. The same was performed for ATPE – one triplet done just with standards in toluene, second triplet was done by standards in acetone.

## 5.9 GC-MS

Aliquots of 1  $\mu\text{L}$  of each sample, standard solutions and calibration solutions were analyzed using a Trace GC Ultra gas chromatograph interfaced with s ITQ 1100 mass detector (both Thermo Fischer Scientific, USA). A Zebron DB5-MS capillary column (30 m x 0.25 mm I.D. and film thickness of 0.25  $\mu\text{m}$  (Phenomenex, USA) was used with helium as the carrier gas at a constant flow rate of 1  $\text{mL min}^{-1}$ . For the analysis of coprostanol, the injector and MS source temperatures were maintained at 250 and 200 $^{\circ}\text{C}$ , respectively. The initial column temperature was set at 150 $^{\circ}\text{C}$ , followed by first ramp at 50 $^{\circ}\text{C min}^{-1}$  to 260  $^{\circ}\text{C}$  and second ramp of 2 $^{\circ}\text{C min}^{-1}$  to 285  $^{\circ}\text{C}$ . The MS was operated in the Full-Scan mode (50-600 m/z). The samples were analyzed in the splitless mode.

For analysis of DEET, cotinine and galaxolide, the injector and MS source temperatures were maintained at 250 and 200  $^{\circ}\text{C}$ , respectively. The column temperature program consisted of injection at 100 $^{\circ}\text{C}$  and hold for 3 min, temperature increase of 10 $^{\circ}\text{C min}^{-1}$  to 220  $^{\circ}\text{C}$ , followed by the temperature rise of 2 $^{\circ}\text{C min}^{-1}$  to 230  $^{\circ}\text{C}$ . The MS was operated in the MS2 mode (191 m/z for 10.8 min; 176 m/z for 12.35 min; 258m/z for 13.90 min. The samples were analyzed in the splitless mode.



## 6 Results

### 6.1 DEET

**Table IV:** The results from GC-MS analysis of the water sample taken on 18.06.2012

| Date: 18.06.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | < LOQ <sup>2</sup>                      |
| ATPE outflow     | ND <sup>3</sup>                         |
| LLE inflow       | 5.24±0.24                               |
| LLE outflow      | 4.92±0.18                               |
| SBSE inflow      | ND                                      |
| SBSE outflow     | ND                                      |

**Table V:** The results from GC-MS analysis of the water sample taken on 12.07.2012

| Date: 12.07.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | < LOQ                                   |
| ATPE outflow     | < LOQ                                   |
| LLE inflow       | 11.67±0.81                              |
| LLE outflow      | 22.78±2.68                              |
| SBSE inflow      | ND                                      |
| SBSE outflow     | ND                                      |

**Table VI:** The recovery DEET recovery from standard extractions

|                 | Average recovery [%] |
|-----------------|----------------------|
| LLE in toluene  | 89.49±7.75           |
| LLE in acetone  | 101.05±7.50          |
| ATPE in toluene | 0                    |
| ATPE in acetone | 0                    |

**Table VII:** Calculated limits of detection

| Type                   | Limit of detection [ $\mu\text{g L}^{-1}$ ] |
|------------------------|---|
| Graham $X_D^\alpha$    | 3.50  |
| Graham $X_D^{\beta 4}$ | 9.62  |
| Miller $X_m^5$         | 4.59  |

<sup>2</sup> < LOQ = the value is lower than the limit of quantification

<sup>3</sup> ND = The value was not detected

<sup>4</sup> (Graham, 1993)

<sup>5</sup> (Miller et al., 2005)

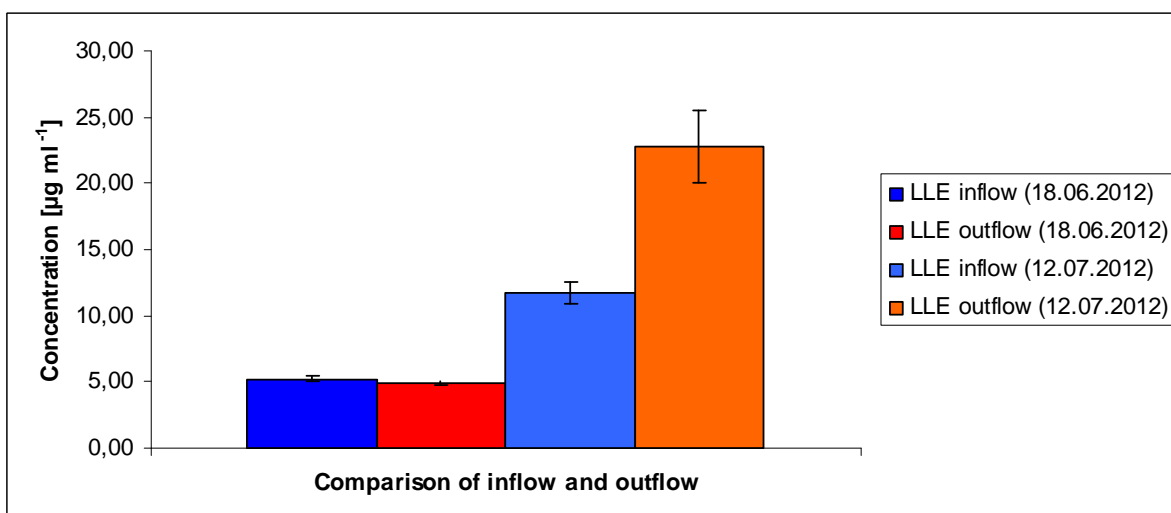


Figure 6: The comparison of the DEET concentrations from inflow and outflow

## 6.2 Cotinine

**Table VIII:** The results from GC-MS analysis of the water sample taken on 18.06.2012

| date: 18.06.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | 40.90±1.48                              |
| ATPE outflow     | ND                                      |
| LLE inflow       | 11.43±0.76                              |
| LLE outflow      | 15.62±1.05                              |
| SBSE inflow      | ND                                      |
| SBSE outflow     | ND                                      |

**Table IX:** The results from GC-MS analysis of the water sample taken on 12.07.2012

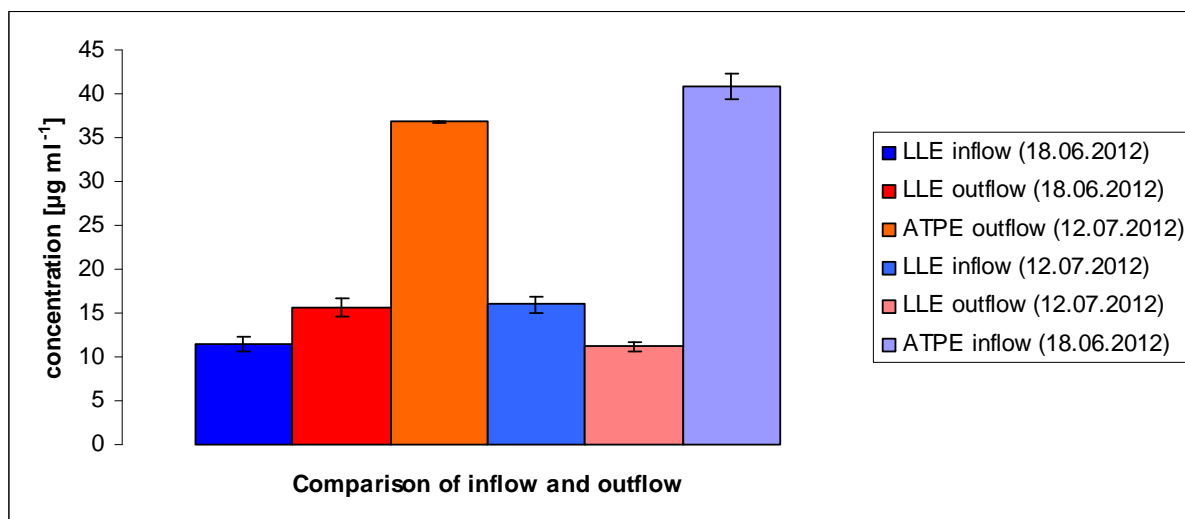
| Date: 12.07.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | ND                                      |
| ATPE outflow     | 36.82±0.11                              |
| LLE inflow       | 15.96±0.87                              |
| LLE outflow      | 11.21±0.55                              |
| SBSE inflow      | ND                                      |
| SBSE outflow     | ND                                      |

**Table X:** The cotinine recovery from standard extractions

|                 | Average recovery [%] |
|-----------------|----------------------|
| LLE in toluene  | 8.79±0.30            |
| LLE in acetone  | 3.53±0.85            |
| ATPE in toluene | 24.30±1.48           |

**Table XI:** Calculated limits of detection

| Type           | Limit of detection [ $\mu\text{g L}^{-1}$ ] |
|----------------|---|
| Graham $X_D^a$ | 4.76  |
| Graham $X_D^b$ | 12.79                                       |
| Miller $X_m$   | 9.85  |



*Figure 7: The comparison of the cotinine concentrations from inflow and outflow*

### 6.3 Coprostanol

**Table XII:** The results from GC-MS analysis of the water sample taken on 18.06.2012

| date: 18.06.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | 34.85±5.41                              |
| ATPE outflow     | 12.66±2.37                              |
| LLE inflow       | 27.03±2.33                              |
| LLE outflow      | 5.52±0.62                               |
| SBSE inflow      | 0.53±0.10                               |
| SBSE outflow     | 0.39±0.05                               |

**Table XIII:** The results from GC-MS analysis of the water sample taken on 12.07.2012

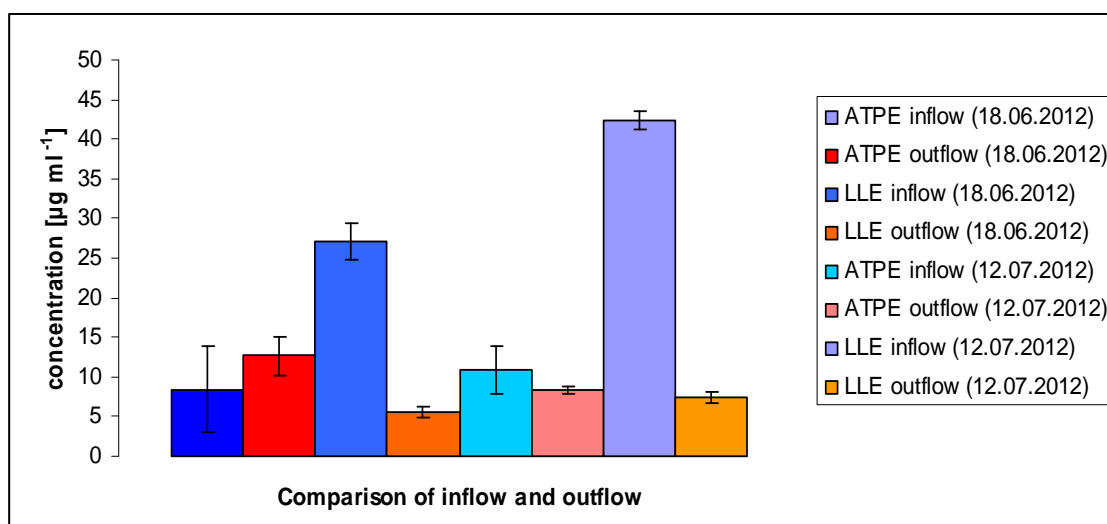
| date: 18.06.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | 10.84±3.07                              |
| ATPE outflow     | 8.39±0.50                               |
| LLE inflow       | 42.46±1.17                              |
| LLE outflow      | 7.41±0.74                               |
| SBSE inflow      | 0.34±0.06                               |
| SBSE outflow     | 0.36±0.08                               |

**Table XIV:** The coprostanol recovery from standard extractions

|                 | Average recovery [%] |
|-----------------|----------------------|
| LLE in toluene  | 93.66±3.43           |
| LLE in acetone  | 86.11±3.44           |
| ATPE in toluene | 30.66±11.05          |
| ATPE in acetone | 33.48±7.71           |

**Table XV:** Calculated limits of detection

| Type           | Limit of detection [ $\mu\text{g L}^{-1}$ ] |
|----------------|---|
| Graham $X_D^a$ | 5.43  |
| Graham $X_D^b$ | 14.58                                       |
| Miller $X_m$   | 10.69                                       |



*Figure 8: The comparison of the coprostanol concentrations from inflow and outflow*

## 6.4 Galaxolide

**Table XVI:** The results from GC-MS analysis of the water sample taken on 18.06.2012

| date: 18.06.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | ND                                      |
| ATPE outflow     | ND                                      |
| LLE inflow       | 11.90±1.43                              |
| LLE outflow      | 8.06±3.69                               |
| SBSE inflow      | 1.62±0.25                               |
| SBSE outflow     | 0.38±0.04                               |

**Table XVII:** The results from GC-MS analysis of the water sample taken on 12.07.2012

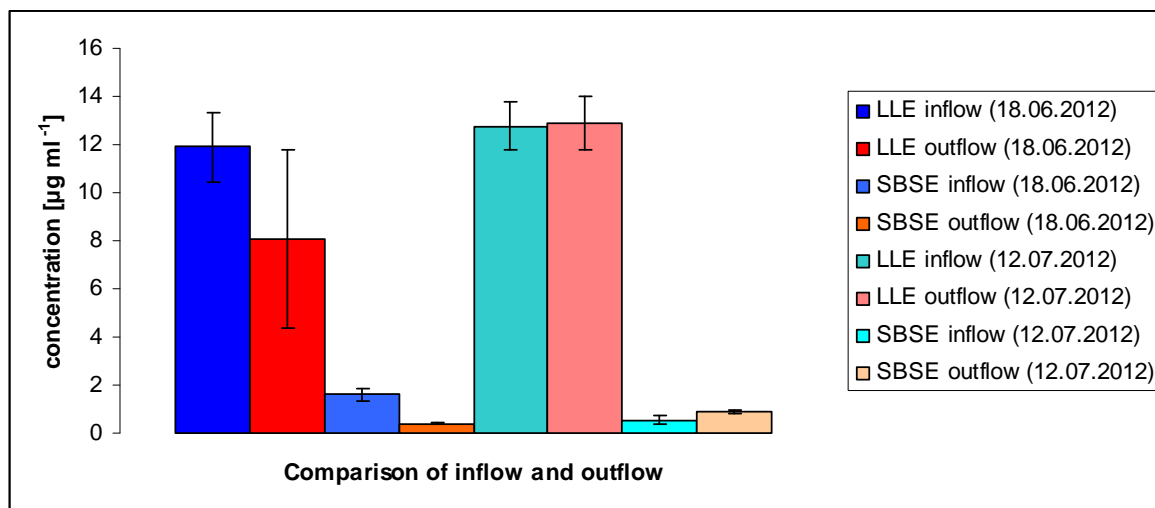
| date: 12.07.2012 | Average amount [ $\mu\text{g L}^{-1}$ ] |
|------------------|---|
| ATPE inflow      | ND                                      |
| ATPE outflow     | ND                                      |
| LLE inflow       | 12.77 $\pm$ 1.00                        |
| LLE outflow      | 12.92 $\pm$ 1.11                        |
| SBSE inflow      | 0.55 $\pm$ 0.17                         |
| SBSE outflow     | 0.90 $\pm$ 0.05                         |

**Table XVIII:** The galaxolide recovery from standard extractions

|                 | Average recovery [%] |
|-----------------|----------------------|
| LLE in toluene  | 88.05 $\pm$ 5.52     |
| LLE in acetone  | 99.29 $\pm$ 4.61     |
| ATPE in toluene | 0.00                 |
| ATPE in acetone | 0.00                 |

**Table XIX:** Calculated limits of detection

| Type           | Limit of detection [ $\mu\text{g L}^{-1}$ ] |
|----------------|---|
| Graham $X_D^a$ | 1.84  |
| Graham $X_D^b$ | 5.16  |
| Miller $X_m$   | 4.65  |



*Figure 9: The comparison of the galaxolide concentrations from inflow and outflow*

## 7 Discussion

The results showed that there is the difference in concentrations of studied substances during the time period. In case of DEET the average amount of inflow on 18.06.2012 was determined by LLE to be  $5.24 \pm 0.24 \mu\text{g L}^{-1}$  whereas the concentration in inflow on 12.07.2012 was  $11.67 \pm 0.81 \mu\text{g L}^{-1}$ . This difference can be caused by lower usage of insecticides in June than in July. The literature values are comparable, according to study performed by Barnes et al (2004), the concentration of DEET in the ground water was ranging from 5.5 to  $13 \mu\text{g L}^{-1}$ . The results with respect to cotinine showed comparable values of the concentration in inflow during the time -  $11.43 \pm 0.76$  and  $15.96 \pm 0.87 \mu\text{g L}^{-1}$  -this could be caused by stable smoker's activity. The literature values, however shows much lower concentrations, Buerge et al. (2008) determined the concentration of cotinine in sewage influent ranging from 780 to  $2650 \text{ ng L}^{-1}$ . These values are highly dependent on the water level and subsequent dilution and also potential degradation of the studied compound by aquatic environment. The coprostanol concentration in inflow on 18.06.2012 and 12.07.2012 was determined by LLE to be  $27.03 \pm 2.33 \mu\text{g L}^{-1}$  and  $42.46 \pm 1.17 \mu\text{g L}^{-1}$  respectively. As the coprostanol can be used as the indicator of faecal pollution, it could be estimated that on 18.06.2012 there was higher water dilution e.g. rainy weather than on 12.07.2012 (Vymazal, 2011). The literature value of coprostanol concentration in the waste water treatment plant was 36–183  $\mu\text{g L}^{-1}$  (Beck and Radke, 2006), which is again comparable to determined results. The concentration is dependent on the density of population along the treatment plant and again on the weather. Concerning galaxolide the concentration was comparable on both dates -  $11.90 \pm 1.43 \mu\text{g L}^{-1}$  on 18.06.2012. and  $12.77 \pm 1.00 \mu\text{g L}^{-1}$  on 12.07.2012 by LLE. The literature values of galaxolide concentration are various, e.g. the concentration in wastewater treatment plant inflow was determined to be  $13.7 \pm 1.5 \mu\text{g L}^{-1}$  (Simonich et al, 2000), which is quite comparable to the results obtained in this study. However, Artola-Garicano et al. (2003) reported the concentration of galaxolide ranging between 1.25 and  $258 \mu\text{g L}^{-1}$ . The concentrations are again dependent on the actual amount of water in the treatment plants.

The comparison of inflow and outflow concentrations is not possible, because we miss the data of concentrations within the flow through and we also do not know how long the water sample spends in the reed bed. This is the issue for further observation.

The second goal of the bachelor thesis was the comparison of extraction techniques used for preparation of samples for GC-MS analysis. The aqueous two phase extraction

(ATPE) was determined to be in general insufficient technique. This method was originally developed for high molecular weight substances and now it is tested also for low molecular weight substances. My results suggest that conditions used in this study were not useful for the extraction of studied compounds. In case of DEET the concentration determined was under the limit of quantification or not detected at all, which was also true for the recovery test using standards in toluene and acetone. In case of cotinine, the results were not stable – in two triplets the compound was not detected and in two triplets it was detected. However, the recovery test gave us higher value than for liquid-liquid extraction –  $24.30 \pm 1.48$  %. Concerning coprostanol the values were again „jumping“ sometimes showing higher yield than LLE, but the recovery test has given the average recovery of  $30.66 \pm 11.05$  % for standards in toluene and  $33.48 \pm 7.71$  % for standards in acetone. The results of ATPE with respect to galaxolide were obvious, because this technique was not working at all. No data of this extraction technique used for sample preparation of studied compounds were found.

Liquid-liquid extraction (LLE) gave high yield with respect to DEET with the average recovery of  $89.49 \pm 7.75$  % for standards in toluene and  $101.05 \pm 7.50$  % for standards in acetone. These values are comparable to the literature values of liquid-liquid extraction of plasma samples and urine samples giving the recovery values of  $84.5 \pm 4.0$  and  $82.3 \pm 4.5$  % respectively (Abu-Qare and Abou-Donia, 2001). In case of cotinine the recovery was  $8.79 \pm 0.30$  % for standards in toluene and  $3.53 \pm 0.85$  for standards in acetone. The literature value of liquid-liquid extraction of cotinine using rat plasma and dichloromethane gave the recovery of  $48 \pm 22$ % (Jung et al., 1999). Coprostanol was also sufficiently recovered with values of  $93.66 \pm 3.43$  % for standards in toluene and  $86.11 \pm 3.44$  % for standards in acetone. The literature value for coprostanol recovery of liquid liquid extraction using non-chlorinated solvents was  $86.75 \pm 4.66$  % (Börjesson et al., 1998). Galaxolide was recovered by  $88.05 \pm 5.52$  % for standards in toluene and  $99.29 \pm 4.61$  % for standards in acetone.

The stir bar sorptive extraction (SBSE) was found to be not suitable for extraction of these compounds. DEET and Cotinine were not detected by this technique and coprostanol and galaxolide were yielded in much lower concentrations. The test of recovery of these compounds was not performed due to lack of material. The fact that DEET was not detected at all is not according to literature, because the method of SBSE coupled to thermodesorption–gas chromatography-mass spectrometry was successfully used for investigation of the insecticides in water samples giving the extraction efficiency of 44 % for DEET (Rodil et al., 2008). This method was also successfully used for analysis of musks (Marcé., 2011).

The retention times of the studied compounds were found to be 10.88, 11.57, 12.44 and 13.98 for DEET, coprostanol, cotinine and galaxolide respectively. The limits of detection according to Graham were 3.50 and 9.62  $\mu\text{g L}^{-1}$  for DEET, 4.76 and 12.79  $\mu\text{g L}^{-1}$  for cotinine, 5.43 and 14.58  $\mu\text{g L}^{-1}$  for coprostanol and 1.84 and 5.16  $\mu\text{g L}^{-1}$  for galaxolide (Graham, 1993) for  $X_D^a$  and  $X_D^b$  respectively. The calculated limits of detection according to Miller were 4.59, 9.85, 10.69 and 4.65  $\mu\text{g L}^{-1}$  for DEET, cotinine, coprostanol and galaxolide respectively.



## **8 Conclusion**

The four compounds – DEET, cotinine, coprostanol and galaxolide were studied during the time period with respect to their concentrations in the inflow and outflow of the constructed wetland with horizontal subsurface flow in Slavošovice. Moreover the three types of extraction techniques for preparation of samples for GC-MS analysis were compared to each other yielding the best yield of DEET, coprostanol and galaxolide using the liquid-liquid extraction. The aqueous two phase extraction gave higher recovery of cotinine however, the technique did not provide stable data. The conditions for GC-MS analysis of the studied compounds were found.

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## 10 List of Appendixes

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## 11 Appendix

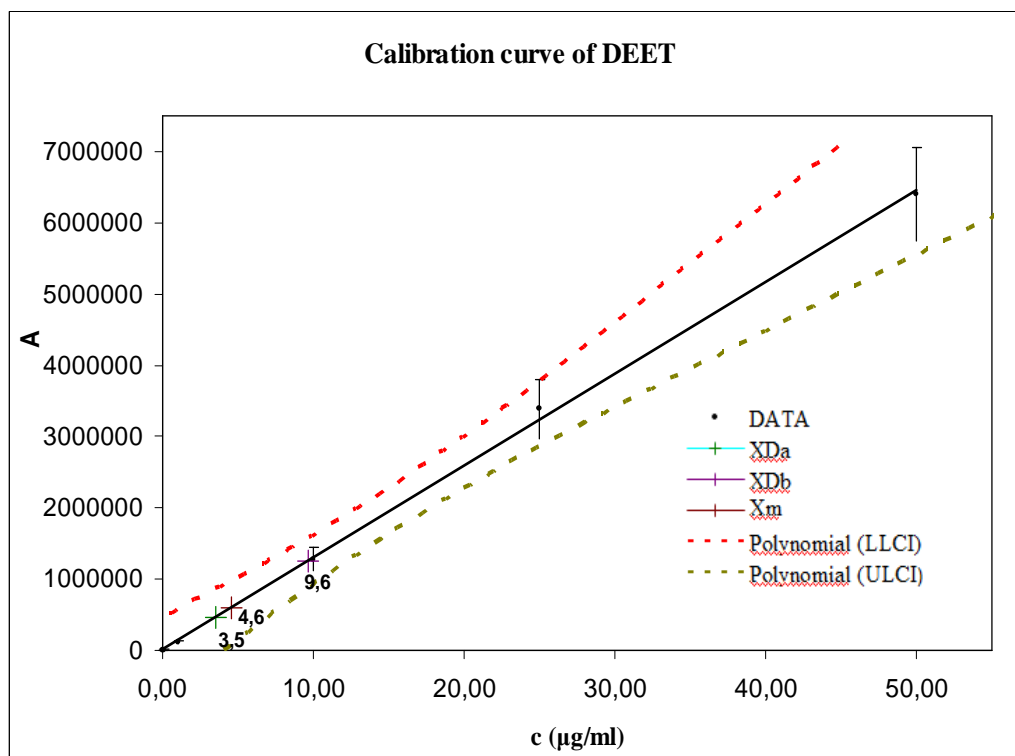


Figure 1: The calibration curve of DEET: LLCI = lower limit of confidence interval; ULCI = Upper limit of confidence interval

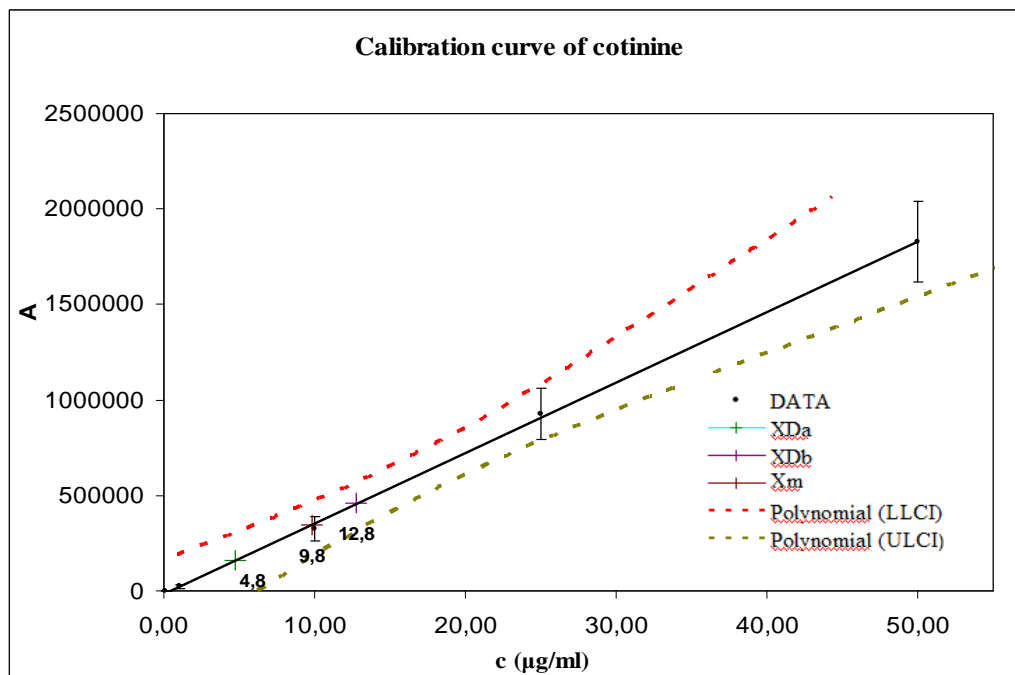


Figure 2: The calibration curve of cotinine: LLCI = lower limit of confidence interval; ULCI = Upper limit of confidence interval

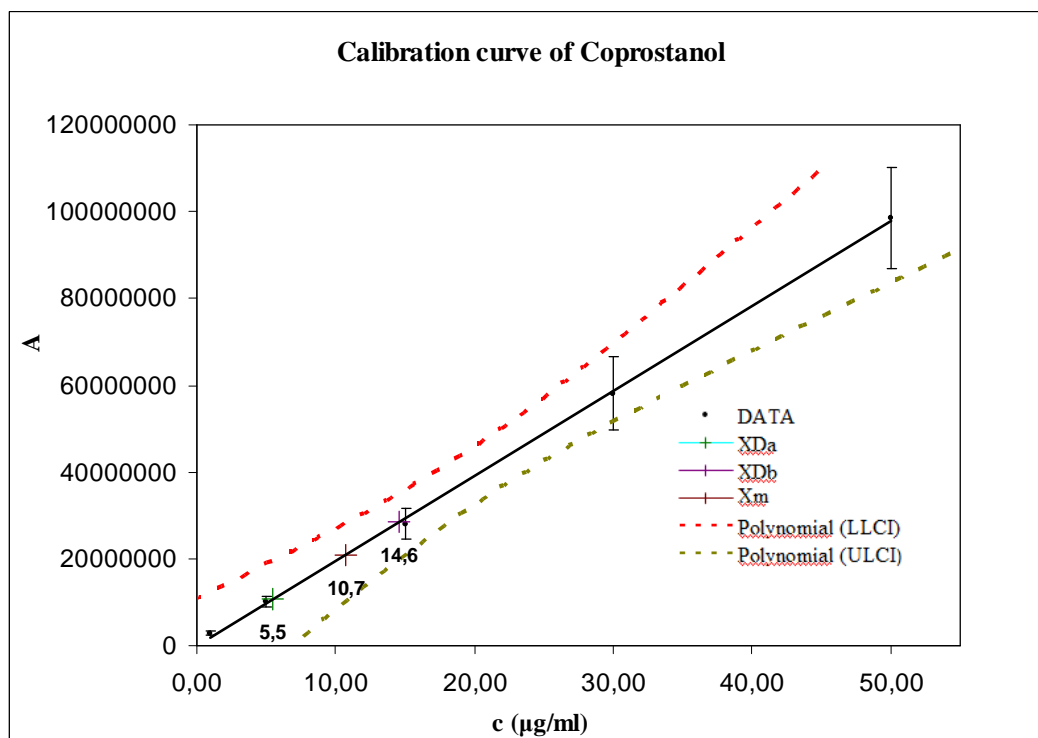


Figure 3: The calibration curve of coprostanol: LLCI = lower limit of confidence interval; ULCI = Upper limit of confidence interval

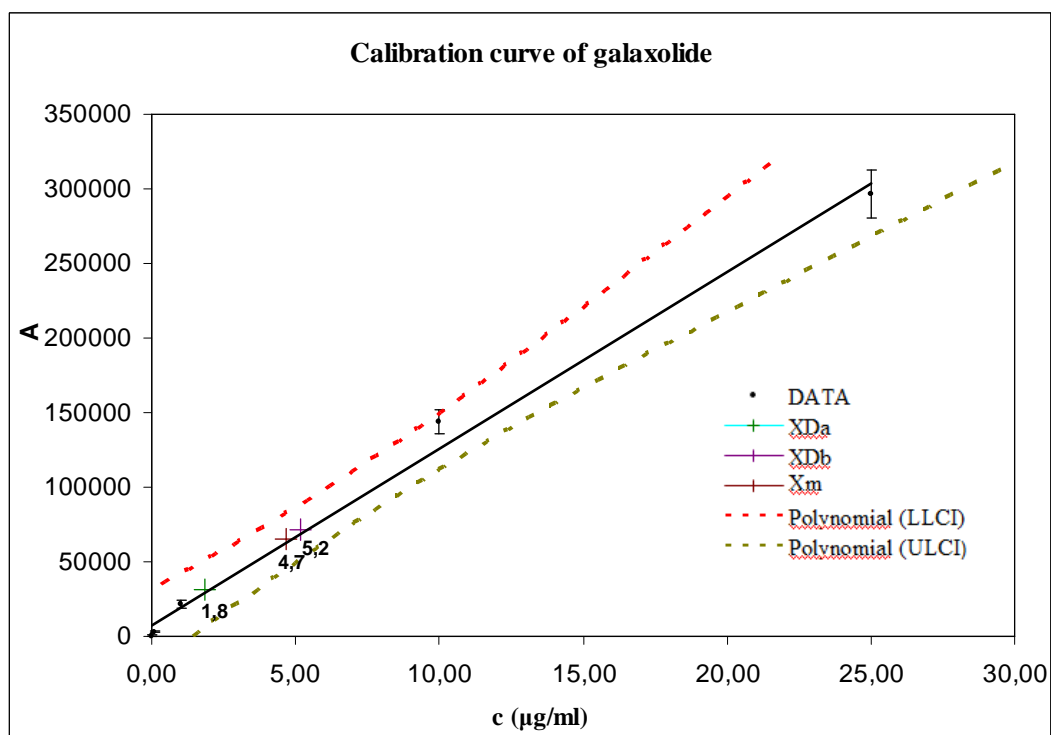


Figure 4: The calibration curve of galaxolide: LLCI = lower limit of confidence interval; ULCI = Upper limit of confidence interval

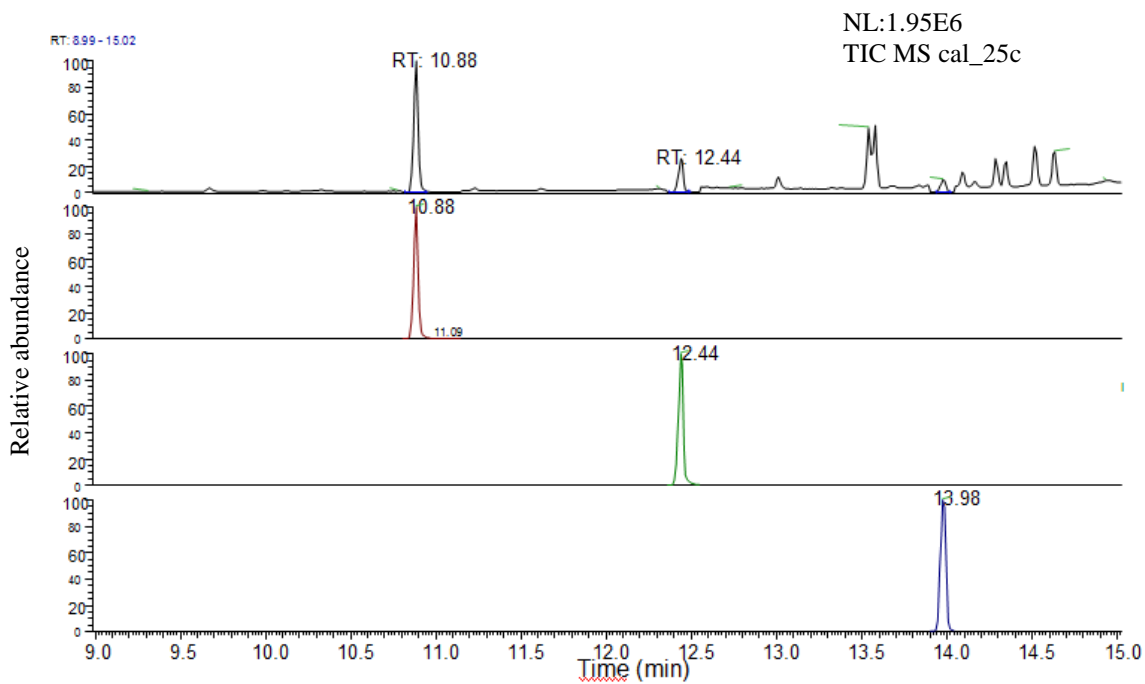


Figure 5: The chromatogram of calibration solution: DEET (RT = 10.88), cotinine (RT = 12.44) and galaxolide (RT = 13.98)

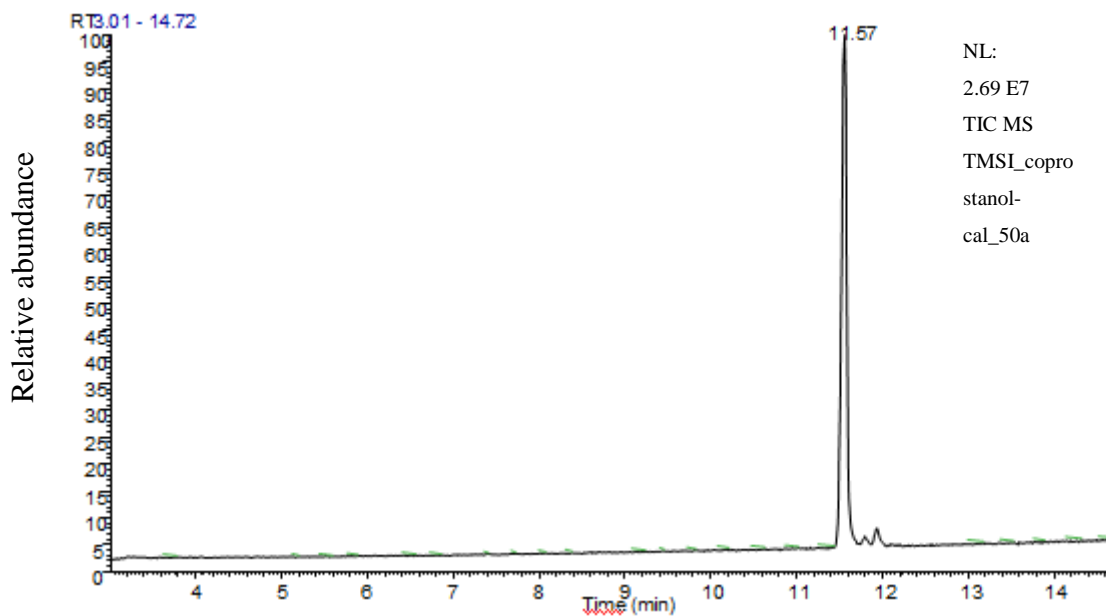


Figure 6: The chromatogram of calibration solution: coprostanol (RT = 11.57)

cal\_25c #578-580 RT: 10.88-10.89 AV: 3 SB: 7 10.98-11.04 , 10.69-10.79 NL: 2.76E5  
T: + c SRM ms2 191.00@cid0.53@cid1.06@cid1.91 [63.00-200.00]

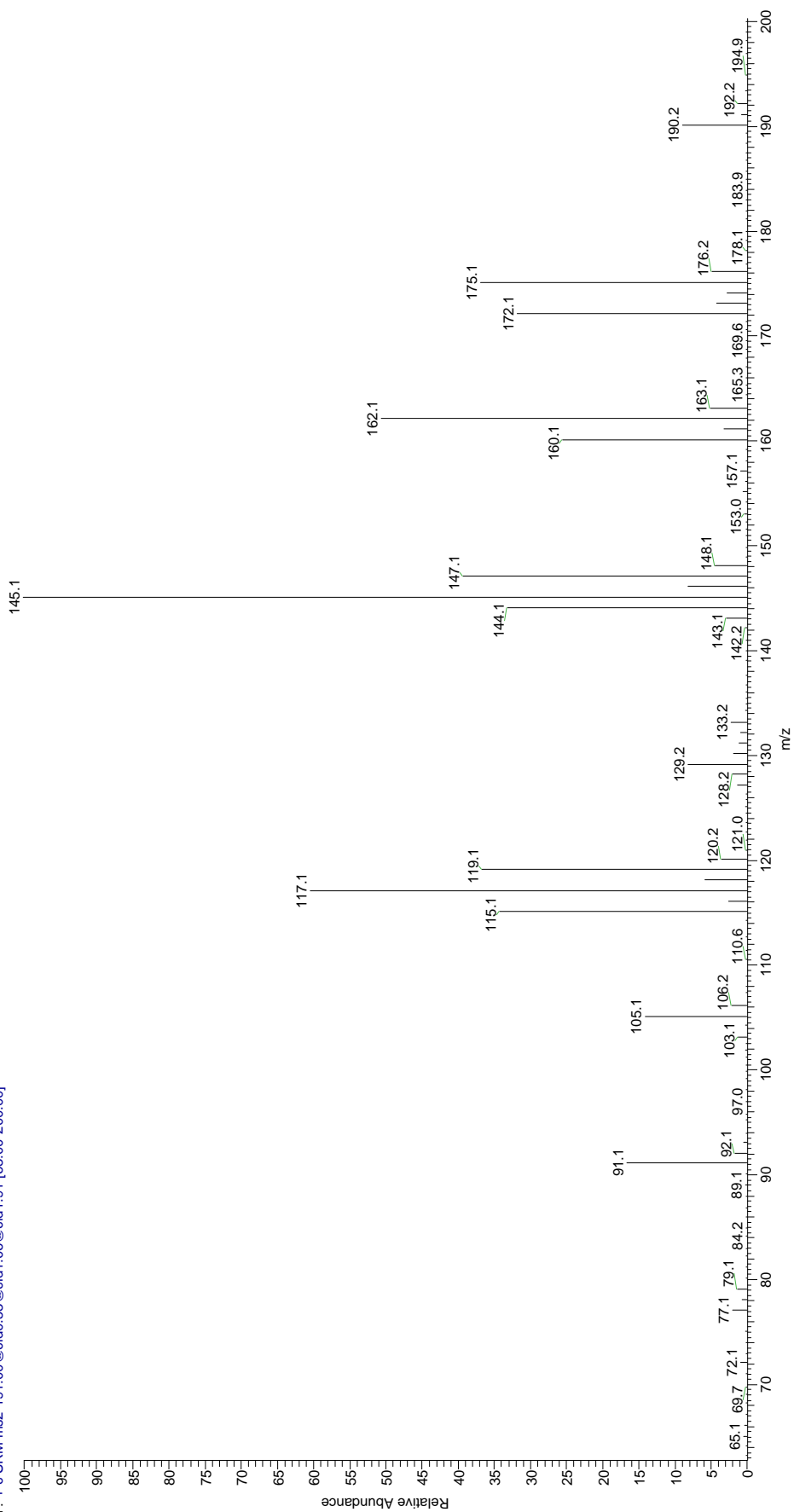


Figure 7: The mass spectrum of DEET

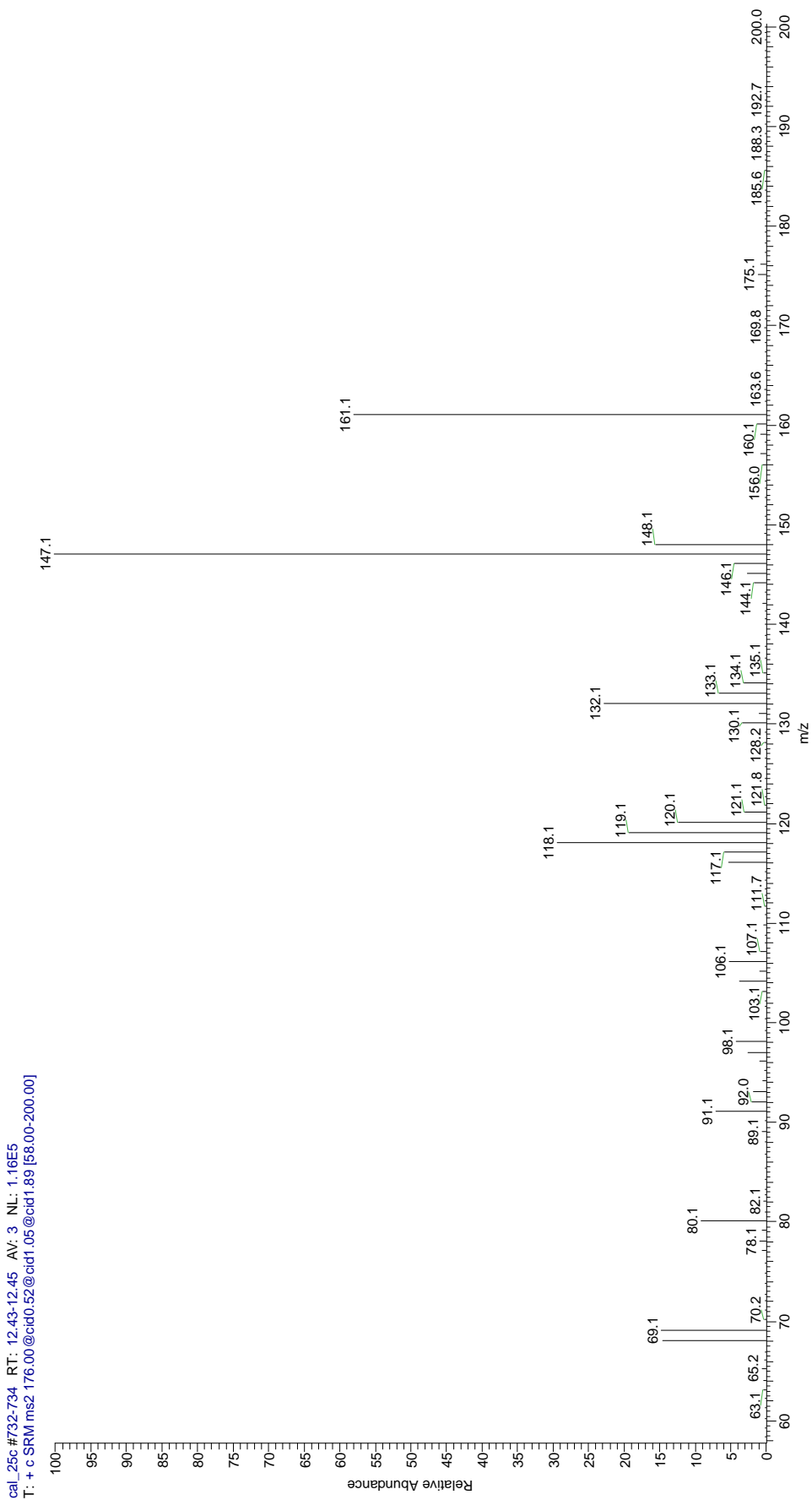


Figure 8: the mass spectrum of cotinine

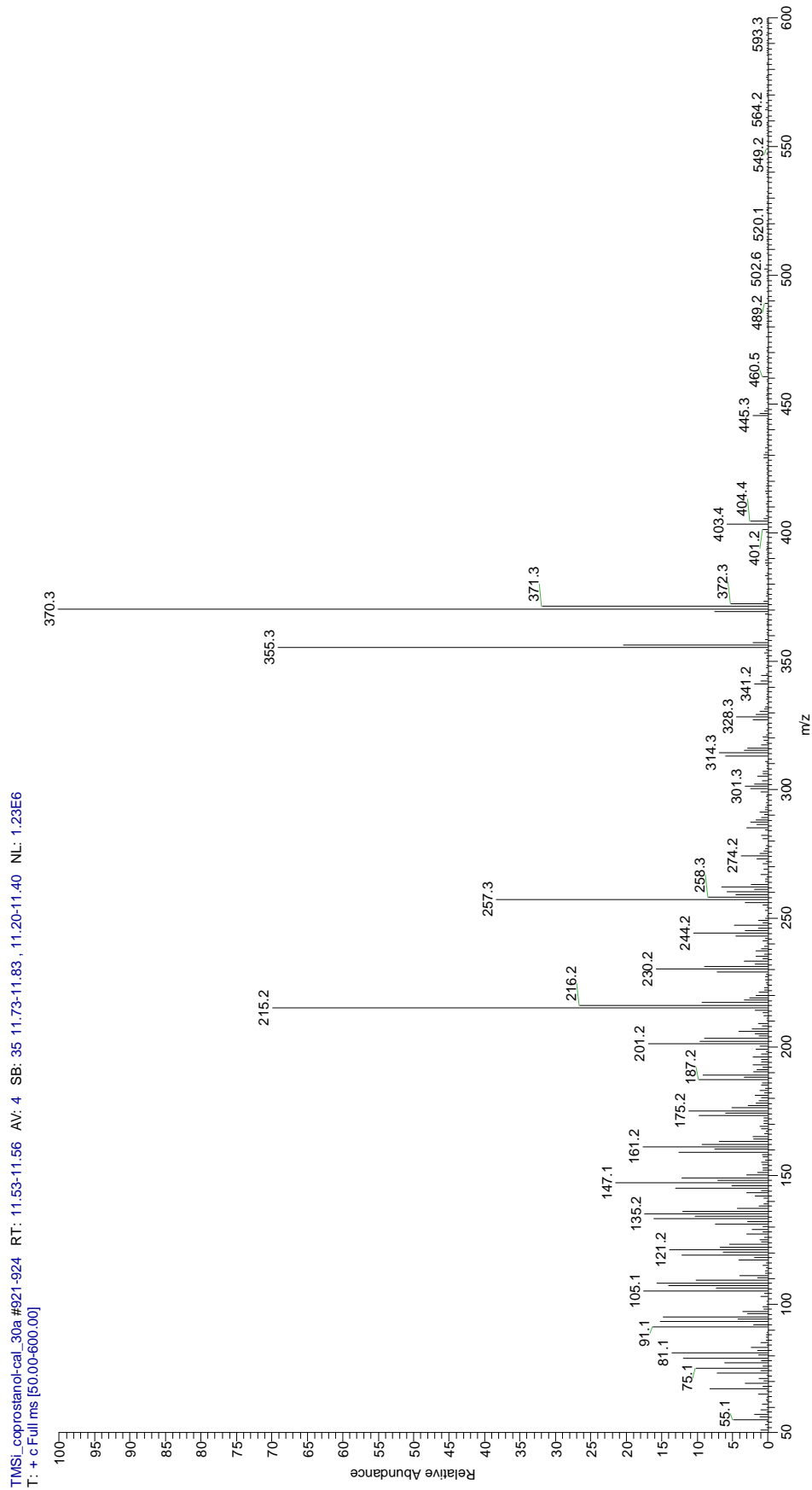


Figure 9: The mass spectrum of coprostanol

cal\_25c #885-887 RT: 13.97-13.99 AV: 3 SB: 5 14.03-14.09 , 13.81-13.92 NL: 1.18E5  
T: + c SRM ms2 258.00@cid0.56@cid1.12@cid2.02 [86.00-300.00]

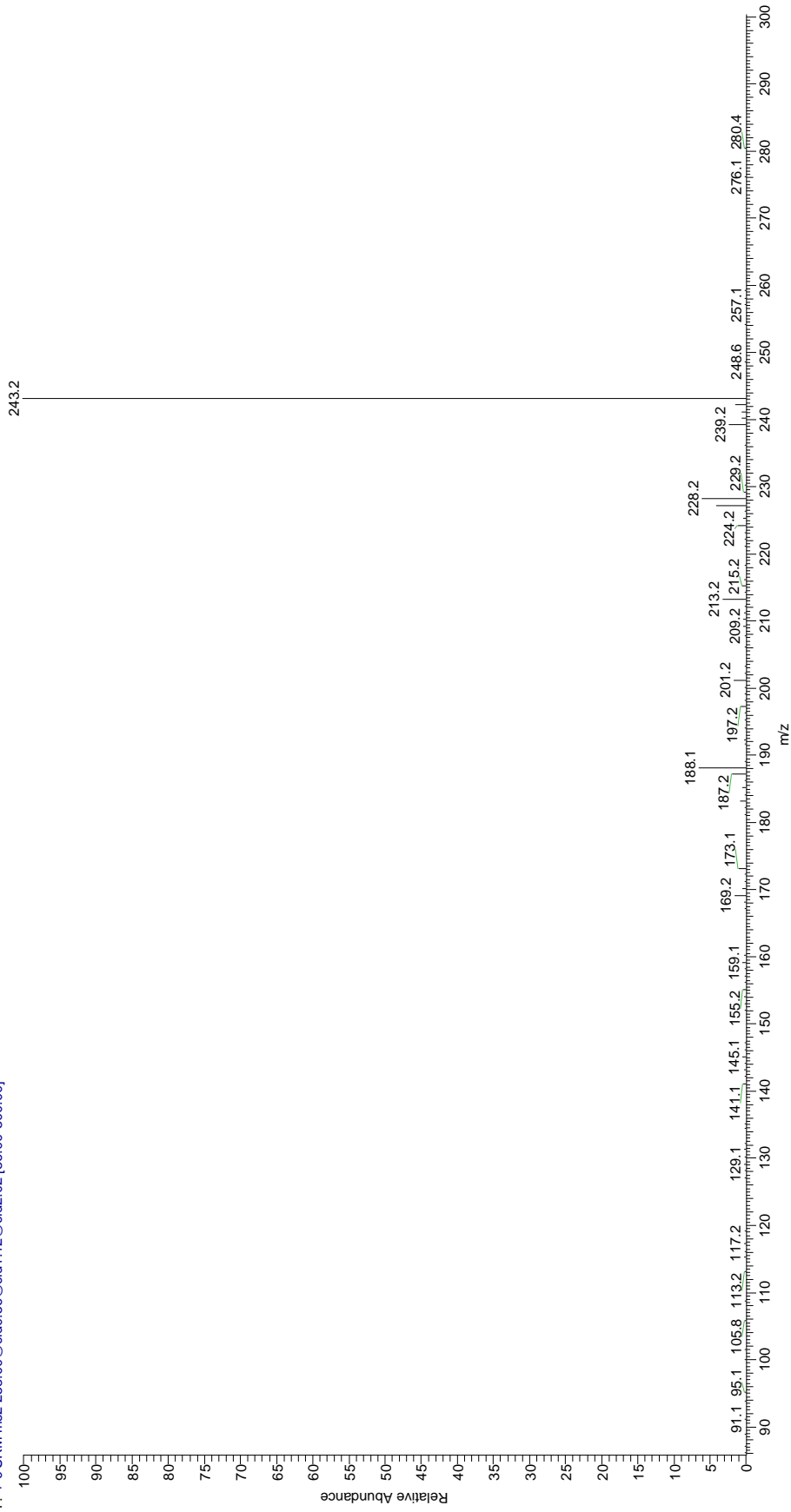


Figure 10: The mass spectrum of galaxolide