Separation and determination of selected organic pharmaceuticals in waters by means of natural flat membranes, GC, HPLC and mass spectrometry

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Table of contents

Abbreviations

AA	Ammonium acetate
9-AC 9	Hydroxymethyl-10-carbamoyl acridan
Ac-SFM	N-4-Acetyl-sulfamethoxazole
API	Atmospheric pressure ionisation
BSA	N,O-Bis(trimethylsilyl)acetamide
C-18	Octadecasilane
CA-HA	Carboxyhydratropic acid
CBZ	Carbamazepine
CBZ-2-OH	2-Hydroxy-carbamazepine
CBZ-3-OH	3-Hydroxy-carbamazepine
CBZ-10-OH	10, 11-Dihydro-10-hydroxycarbamazepine
8-CCA	8-Chlorocarbazole-1-aceticacid
β-CD	β-Cyclodextrine
CH_2N_2	Diazomethane
CID	Collision induced dissociation
CX-IBU	Carboxyibuprofen
DCF	Diclofenac
Diol-CBZ	10,11-Dihydro-10,11-dihydroxycarbamazepine
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact ionization
EP-CBZ	10,11-Dihydro-10,11-epoxycarbamazepine
ESI	Electrospray ionization
EtOAC	Ethyl acetate
GC	Gas chromatography
Glucu-SFM	N-1-Glucuronide sulfamethoxazole
GW	Ground water
HO13	Hohlohsee13
HPLC	High performance liquid chromatography
HUS	Humic substances
IBU	Ibuprofen
IDL	Instrument detection limit
IMINO	Iminostilbene
i.d.	Internal diameter
ISAS	Institute for analytical sciences
K_a	Dissociation constant
K_{ow}	Distribution coefficient octanol/water
LC	Liquid chromatography
Lichrolut EN	Ethylvinylbenzene-di-vinylbenzene-copolymer
LOQ	Limit of quantitation
Me	Methyl group
MDL	Method detection limit
MeOH	Methanol
Min	Minute
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cutoff
m/z	Mass to charge ratio
n	Number of samples

n.d.	Not detected
NIST	National Institute of Standards & Technology (USA)
Oasis HLB	Poly (divinylbenzene-co-N-vinylpyrrolidone)
OECD	Organization for Economic Co-operation and Development
OH-DCF	4`-Hydroxy-diclofenac
OH-IBU	2-Hydroxyibuprofen
PCB 169	3,3',4,4',5,5'-hexachlorobiphenyl
PEG	Polyethylene glycol
PFBB	Pentafluorobenzyl bromide
PH	The negative logarithm of the hydrogen ion (H^+) concentration
PhACs	Pharmaceutically active compounds
PTFE	Poly (tetra fluoro ethylene)
r^2	Regression coefficient
R.I.	Refractive index
RSD	Relative standard deviation
RPM	Rotation per minute
R_t	Retention time
SDS	Sodium dodecylsulfate
SFM	Sulfamethoxazole
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
STD	Standard
STP	Sewage treatment plants
SW	Surface water
SWM	Surface water affected by the run off of a wastewater sewage plant
TIC	Total ion current
TMS	Trimethylsilyl group
UV	Ultraviolet
VM	Venner Moor
V/V	Volume/volume
W/W	Weight/weight
WW	Wastewater

1. Introduction and aim of the study

It was reported in the last decade in many investigations about the emerging environmental residuals in aquatic compartments, especially those of toxic/carcinogenic pesticides and industrial intermediates displaying persistence in the environment. Other diverse groups of bioactive chemicals receiving comparatively little attention as potential environmental pollutants include the pharmaceuticals and active ingredients in personal care products [1-6].

In this work pharmaceutical compounds and their metabolites are collectively termed pharmaceutically active compounds (PhACs), though not all metabolites must be active.

The occurrence of the PhACs in the aquatic environment has been investigated in several studies in many countries. More than 80 PhACs from various prescription classes have been detected up to the μg/L level in sewage, surface water and ground water [1].

1.1. Pharmaceuticals and their effects on the aquatic environment

Sources and pathways
Depending on their use, pharmaceuticals enter the environment on different pathways, as outlined in (Fig. 1.1) [7]. Drugs applied by human are excreted via urine and faeces as a mixture of metabolites and/or as the unchanged substance to enter the sewer system. The waste drugs unused are assumed to be disposed of in the sewer system as surplus medical substance.

Subsequently, they are released via the effluents of sewage treatment plants (STPs) into the aquatic environment. The proportion of a drug that is retained in sewage treatment either due to transformation or by adsorption to sludge strongly depends on its chemical structure and physico-chemical properties, but also on the specific conditions within the respective plant. Water temperature, residence times corresponding to flow rates, dilutions with rainwater and sludge age (and thus adaptation of microbial communities) were found to have influence on elimination efficiencies [8-9]. If the drugs are not eliminated in STPs, they may enter the aquatic environment and eventually reach drinking water Fig. 1.1. Observed elimination rates ranged from more than 80 % for ibuprofen to less than 10 % for carbamazepine [2,10]. Comparison of the elimination rates of different municipal STPs gives no reliable results due to varying influent concentrations and operating parameters. [11]

In many cases, veterinary pharmaceuticals are directly released into the environment by its use in agriculture, the dispersion of manure from treated livestock on fields or the therapeutic treatment of livestock on meadows [1,7,106].

Effects
Recent European studies have revealed the presence of a wide array of non-hormonal, non-antibiotic pharmaceuticals and antiseptics in surface waters, STP effluents and even in drinking water, often at a level comparable to traditionally recognized “priority pollutants” [1].

From these studies, we can recognize the magnitude of the problems caused by an increase of the concentrations of some non-degradable PhACs in aquatic environment during the last years. Little information is available on the long-term effect of the active substance on organisms in the aquatic and terrestrial environment [1]. High concentrations of some compounds, i.e. in the mg/L range have been found to produce effects in environmental

organisms. However, an effect on Daphnia, algae and bacteria has also been demonstrated using low concentration in chronic tests [1].

Since 1997 the interest in the occurrence and behaviour of pharmaceuticals in the aquatic environment has significantly increased [2-3, 6-7, 12-14]. One motivation for this attention is the fact that these chemicals are designed to trigger specific biological effects. Thus, they can be expected to interfere with the respective receptors, enzymes or hormonal systems of unintentionally exposed organisms.

In the case of antimicrobial agents, the possible maintenance and spread of bacterial resistance is of major concern. In industrialized countries most human use antimicrobials and other pharmaceuticals, which reach the aquatic environment unchanged or transformed, mainly via discharge of effluent from municipal STPs. The residual concentrations of these bioactive compounds in the treated effluents depend on their removal during wastewater treatment. They can potentially pose a hazard for aquatic organisms if the removal is incomplete. In addition, exposure via sewage sludge disposal on land could cause a hazard for soil organisms.

General drug classification
Human medicine based on its medical function is classified in analgesics and anti-inflammatories, antibiotic/bacteriostatics (antibacterial drugs), antiepileptics, beta-blockers, blood lipid regulators, contrast media and cytostatic drugs.

1.2. Aim of the study

To solve environmental problems sensitive analytical methods based on an enrichment step, chromatographic separation and mass spectrometric determination with and without derivatization should be developed. In this study carbamazepine (CBZ), diclofenac (DCF), ibuprofen (IBU) and sulfamethoxazole (SFM) were chosen based on their high application quantity in medicine and high concentrations found in aquatic environment in previous studies and further criteria are their great varieties of physical and chemical characteristics, such as pK_a value and polarity. That means, for analytical determination and quantification mass spectrometric methods must be coupled to both a GC and a LC separation. So the developed methods should allow application for many residues of drugs considering their features.

Specifically, the aims of the study were:

1. Within the enrichment procedures the applicability of particular natural flat membranes from animal intestines should be tested to discharge drug residues in water treatment and in sample preparation for the analytical determination of selected target drugs and their metabolites (Table 1.1).

2. Develop a sensitive analytical methods based on mass spectrometry for determination of the target drugs and some of their main metabolites.

3. The fate of the selected drugs and some of their main metabolite should be investigated in surface and ground water under certain pilot model systems such as batch and biofilm reactors applying the developed methods.

1.3. Introduction of the drugs under study

In order to get an impression, how many tons of the selected drugs (Table 1.1) are used each year as human medicine, T. Ternes estimated the sold quantities of the selected pharmaceuticals in 1997 in Germany in tons [15]: CBZ 80, DCF 75, IBU 180 and SFM 60.

Table 1.2 gives an overview of the concentration of the targeted drugs as reported in the literature for different water sources in the last years.

Skimming the dates in Table 1.2, notable differences can be observed among diverse studies and countries. These can be attributed to many reasons, such as the consumption pattern and rates, elimination rates in different STPs, operating parameters and seasonal differences.

1.4. State of the art in drugs analysis of aquatic samples

The measurement of trace concentration of pharmaceuticals in the aquatic environment has paid much attention in the last years, but the problem was only a small number of species can be analysed because there is a lack in reliable analytical techniques. The conventional analytical methods often used have a lack in sensitivity and specificity needed to quantify the subpart per billion concentrations of pharmaceuticals typically present in environmental samples. Furthermore, many pharmaceuticals containing functional groups which render the compounds difficult to extract from water or even to measure. In addition, many methods are subject to significant matrix interference when used to analyse wastewater effluent.

As a result of the widely varying properties of pharmaceuticals no single method or instrument is capable of measuring all of the compounds of interest. In fact, accurate and precise measurement of pharmaceuticals and many other organic compounds often require compromise methods focused on a small number of compounds with similar properties.

Recent improvements in analytical techniques have expanded the compound range that is amenable to specific identification. Using these improved tools, much has been learned about the occurrence, fate and transport of organic pharmaceuticals and many other pollutants in the aquatic environment.

Because water samples, in particular wastewater samples, usually contain a high loading of organic material and suspended particles, filtration is the first step of sample preparation. The filtration step is particularly necessary when a subsequent extraction based on solid-phase extraction (SPE) should be performed, because suspended solids can easily clog the adsorbent bed.

In fact, there are many techniques used for sample pre-concentration applied in environmental analysis as liquid-liquid extraction (LLE), solid phase extraction (SPE) and membrane extraction (ME).

SPE is now the most common sampling technique in environmental, pharmaceutical, clinical, food and industrial analysis. Over time various sampling formats and sorbents have been developed to facilitate a convenient processing of different sample types and to extend the scope of the application.

Silica-based chemically bonded sorbents are the most widely used sorbents for SPE but are unsuitable for some applications. Silica-based sorbents contain a low concentration of ionised

silanol groups capable of retaining basic solutes by an ion-exchange mechanism [16]. Silica-based sorbents are unstable at extreme pH values pH<2 or >8. Porous polymer sorbents offer a high potential in solving problems. They are, in general, stable throughout the full pH range and do not possess ionised silanol groups. Modern porous polymer sorbents are copolymer of styrene and divinylbenzene processed to enhance their properties for SPE [16]. While with C18-silicas retention is achieved by van der Waals forces and eventually by hydrogen bonding between residual silanol groups of the silica sorbent base and functional groups of the analyte, polymer sorbents additionally offer π-π interactions.

The polymeric sorbents have been successfully used for the extraction of the whole range of organic contaminants [93-94]. They proved to be especially suitable for medium to high polar substances, where they showed substantially higher recovery rates than alkyl-silica sorbents [95-96].

From a chemical point of view, pharmaceuticals comprise a complex variety of structure increments, often combining moieties of different polarities in one molecule. A common feature of most substituents of pharmaceuticals is their hydrophilic character. Hydroxy-, carboxy- and amino groups are frequent constituents of pharmacological active substances, necessary either for the intended effect or for the transport to the place of action.

The most common methods of quantifying pharmaceuticals in surface and waste waters involve the use of hyphenated techniques such as GC/MS or HPLC/MS, however, even those sophisticated methods need efficient sample pretreatment to minimize effects of the sample matrix and to enrich the analytes [16-17].

For GC methods, it is often necessary to derivatise the compounds prior to analysis. For HPLC/MS methods, sample clean up or selective extraction may be used to control interferences from organic matter [92]. For both techniques, MS/MS is often used to control interferences from the organic matter present in the water samples.

GC/MS is the method of choice for semi-acidic, phenolic and non-polar target analytes with or without derivatization yielding better separations and lower detection limits. A further advantage for the EI ionization technique used mostly in GC/MS is the presence of the NIST database, while other ionization methods such as chemical ionization (CI) or atmospheric pressure ionization usually results in a quasimolecular ion only. The nature of these ions depends on the reactant gas in GC-MS or on the eluent/buffer composition and ionization conditions in LC-MS. In a similar way LC/MS can successfully be used in environmental analysis of drugs and their metabolites in water resources. This technique provides the opportunity especially for polar, unstable and high molecular mass compounds. Further advantages in comparison to GC/MS are the reduced run time and the fact that derivatization becomes redundant. However, lower resolution and especially the suppression of signals in the electrospray interface by matrix impurities are responsible for limitation of the application.

1.5. State of the art with regards to the scope in water treatment

Residues of pharmaceuticals, their active compounds and their metabolites can be found in all aquatic environments. As a consequence, there is an urgent need to improve the purification of water and wastewater and to monitor the drug input of the different waterways, particularly of surface and groundwater by means of sensitive chromatographic methods.

Organic contaminants present in municipal wastewater, such as pharmaceuticals, may be removed or transformed by a variety of mechanisms. In conventional STPs, pharmaceuticals can be removed by sorption to particles or by biotransformation [101]. Sorption of pharmaceuticals to particles present in wastewater treatment plants can occur either via hydrophobic or electrostatic interactions, e.g. ion exchange, surface complexation [101]. For hydrophobic interaction, the octanol/water partition coefficient is a good predictor of the affinity of the compound for the solid phase. Under the condition encountered in conventional wastewater treatment plants, only those compounds with an octanol/water partition coefficient greater than approximately 100 will be removed to an appreciable degree [18]. A few pharmaceuticals meet this criterion. Therefore, we do not expect a substantial removal of pharmaceuticals by this mechanism. Sorption of pharmaceuticals via other interactions usually requires the presence of acidic, phenolic, or amino functional groups. Although many of the pharmaceuticals contain such functional groups, removal via these mechanisms is not expected to be significant under the conditions encountered in municipal wastewater [101]. Removal of pharmaceuticals also can occur via biotransformation. Available data from full-scale STPs suggest that certain compounds, such as IBU, are readily degraded while other compounds, such as CBZ are removed to a much smaller extent [2].

Conventional treatment systems require a large amount of space, a long treatment time and are often unable to produce effluent that meets quality levels needed for discharge because a lot of organic compounds are not eliminated during the cleaning process. Therefore, a need for other technologies is obviously which are more efficient than the standard primary and secondary treatment processes currently utilised.

Further improvement of water quality after conventional secondary treatment is achieved through tertiary treatments such as sand or activated carbon filtration, nitrification / denitrification, coagulation flocculation, membrane filtration, electrodialysis and reverse osmosis [99,100].

Though many separation procedures both for technical water treatment and for analytical purpose as well have been applied, an overall satisfying method is still missing. Meanwhile, technical solid membranes have led to a standard method for water cleaning processes. But synthetic flat membranes with a favourable pore size often have problems with clogging caused by particles or dissolved macromolecular substances [99]. Moreover, the operation cost in a technical way is still substantial.

Therefore, this work focused on the investigation of other kind of membranes for the depletion of target drugs in water treatment and for sample preparation prior to laboratory analysis.

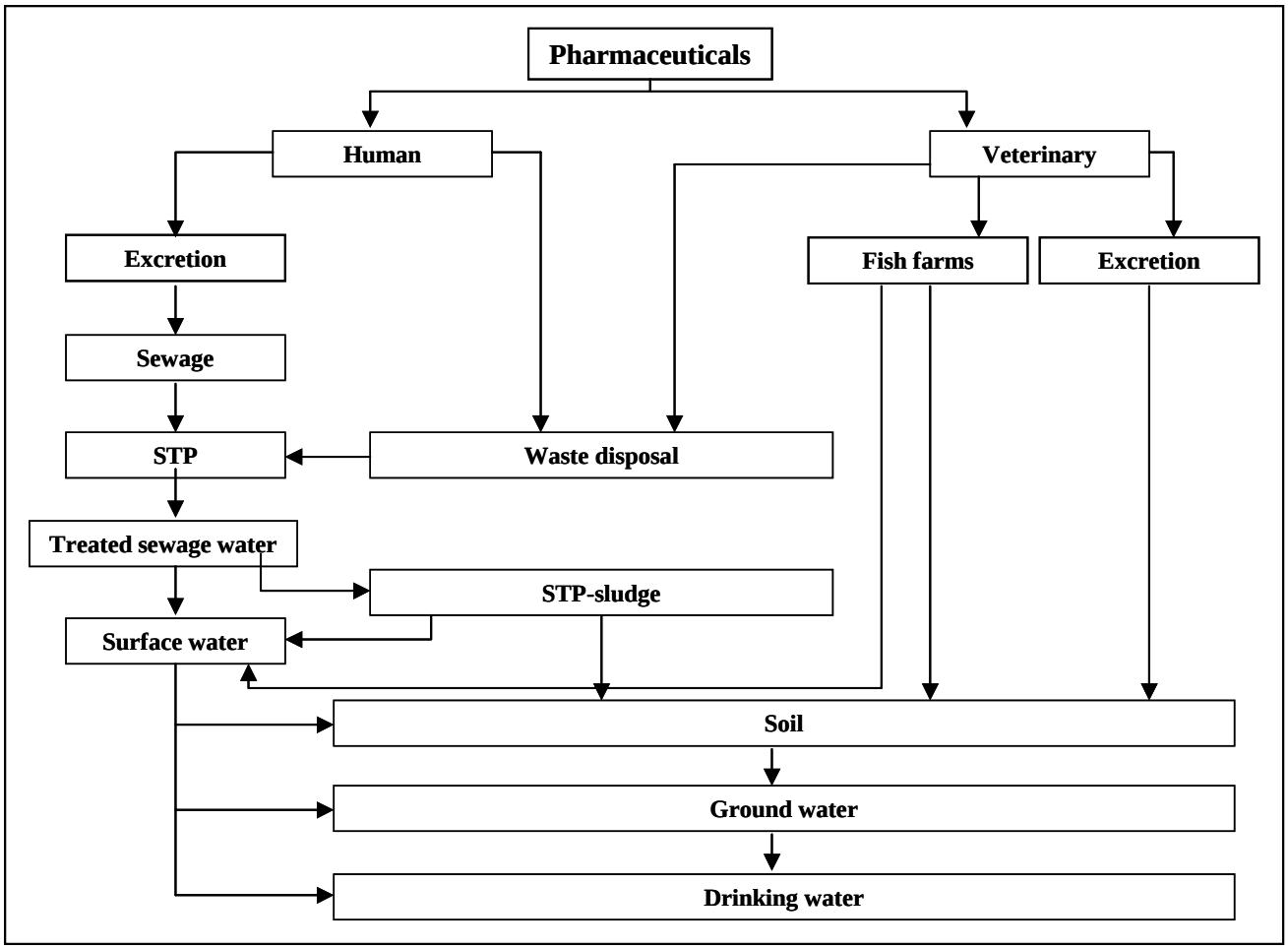
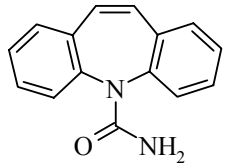
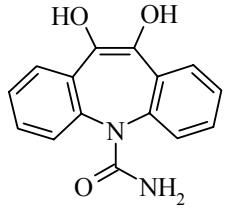
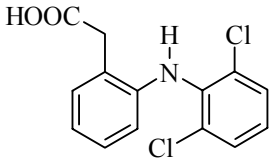
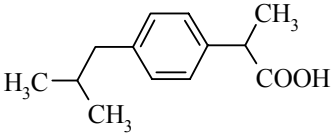
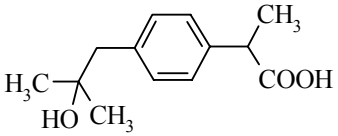
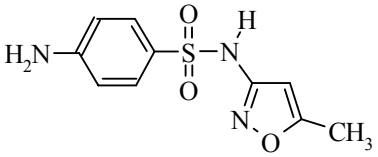
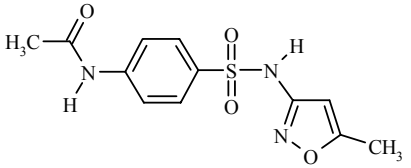
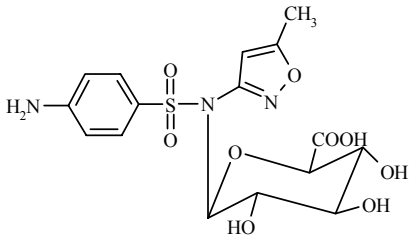


Fig. 1.1: Sources, distribution and sinks of pharmaceuticals in the environment [7]

Table 1.1: pK_a, $logK_{ow}$, structure and application of selected pharmaceuticals and some of their main metabolites

Analyte	Abbreviation	pK_{a1}/ pK_{a2}	Log K_{ow}[98]	Chemical structure	Application
1. Carbamazepine	CBZ	13.94[98]	2.67		Antiepileptic
2. 10,11-Dihydro-10,11-dihyd-roxycarbamazepine	Diol-CBZ*	-	-		-
3. Diclofenac	DCF	4.15[97]	5.44		Anti-inflammatory
4. Ibuprofen	IBU	4.91[97]	3.72		Anti-inflammatory
5. 2-Hydroxyibuprofen	OH-IBU*	-	-		-
6. Sulfamethoxazole	SFM	5.7[20] 1.8[21]	-0.38		Antibiotic

Analyte	Abbreviation	pK_{a1}/ pK_{a2}	Log K_{ow}	Chemical structure	Application
7. N-4-Acetylsulfamethoxazole	Ac-SFM*	5.0 [20]	-		-
8. N-1-Glucuronide-sulfamethoxazole	Glucu-SFM*	-	-		-

*: Synthesised at the University of Paderborn [M. Grote, M. Borges, K. Borchert, unpublished]

Table 1.2: Survey of the concentrations of target pharmaceuticals and some of their main human metabolites detected in different water sources as reported in literature

Analyte	WW-Influent [µg/L]	Effluent-STP [µg/L]	SWM [µg/L]	Rivers [ng/L]	GW [ng/L]	TW [ng/L]
DCF	3.02[6]	0.359-28.4[*] 0.81[2]	0.026-0.194[*]	489[31] 2298[34] 20-150[33] 150[2]		1-6[31]
IBU	3.4[22] 1.5[6]	1.885-24.6[*] 0.37[2]	0.064-0.790[*]	139[31] n.d.-80[33] 70[2]		
OH-IBU	0.92[6]					
CBZ	6.3[22] 0.368[24] 0.635[6]	0.426[24] 2.1[2]	0.020-0.650[*]	250[30] 30-250[33] 250[2]	0.5-7.8[7]	
Diol-CBZ **EP-CBZ** **CBZ-2OH** **CBZ-3OH** **CBZ-10OH**	1.571[24] 0.047[24] 0.121[24] 0.094[24] 0.0085[24]	1.325[24] 0.052[24] 0.132[24] 0.101[24] 0.0093[24]	0.002-0.002[*] n.d.[*] n.d.[*] n.d.[*] n.d.[*]			
SFM	0.243-0.871[22] 343[23]	0.008[*] 0.352[23]	0.099[*]	1000[32]		
Ac-SFM	0.518[23]	0.082[23]				

WW : wastewater
SWM : surface water adjacent to discharge of effluents from STP
GW : ground water
TW : tap water
EP-CBZ : 10,11-dihydro-10,11-epoxycarbamazepine
CBZ-2OH : 2-hydroxycarbamazepine
CBZ-3OH : 3-hydroxycarbamazepine
CBZ-10OH : 10,11-dihydro-10- hydroxycarbamazepine
n.d. : not detected
* : [22, 24-29, 81]

2. Fate of the selected drugs after medical application

The fate of the drugs from medical applications should be evaluated because metabolism can lead to produce new and possibly more toxic species [36]. Drug metabolites have special importance as environmental pollutants, because they are known to be the main excretion products of most active pharmaceuticals. A few data are available in literature concerning the fate and effects of the drugs after the medication.

To answer the question for the fate of the drugs, we have to consider different pathways. First in the human, the major route in human metabolism leads to a series of compounds in varying concentrations [37]. Other drugs have one or two major metabolic pathways that dominate their metabolism, but several minor pathways can produce at least a metabolite too. After ingestion most drugs undergo substance-specific metabolisation distinguished between phase I and phase II metabolites. Phase I reactions usually include oxidation, reduction or hydrolysis, and the products are often more reactive and sometimes more toxic than the respective parent compounds [7]. Phase II reactions involve conjugation mainly with glucuronic or sulfuric acid, but also with acetic acid, glutathion and taurine. Both, phase I and phase II metabolisation renders the parent compound more water soluble [38]. While phase I metabolites may also possess a pharmacological activity that sometimes is even higher than that of the parent drug [39], phase II metabolites are usually inactive. During sewage treatment and in manure cleavage of the conjugates was observed [7]. Secondly in water environments, the degradation might be caused by enzymatic activities, hydrolysis or photodegradation. An other possibility for the metabolism could happen during the biological treatment in the STP induced by biodegradation as described in pilot systems for IBU by C. Zwiener et al. [35].

2.1. Fate of the target drugs in human bodies

CBZ

Thirty three metabolites of CBZ have been identified from human and rat urine [40]. The main metabolic pathway of CBZ is oxidation to EP-CBZ, then hydration to Diol-CBZ and conjugation of Diol-CBZ with glucuronide (Fig. 2.1). The hydrolysis of EP-CBZ to Diol-CBZ is catalysed by microsomal epoxide hydrolase [41]. Some pathways include the oxidation to 9-hydroxymethyl-10-carbamoyl acridan (9-AC), CBZ-2OH, CBZ-3OH and CBZ-10OH [42-43].

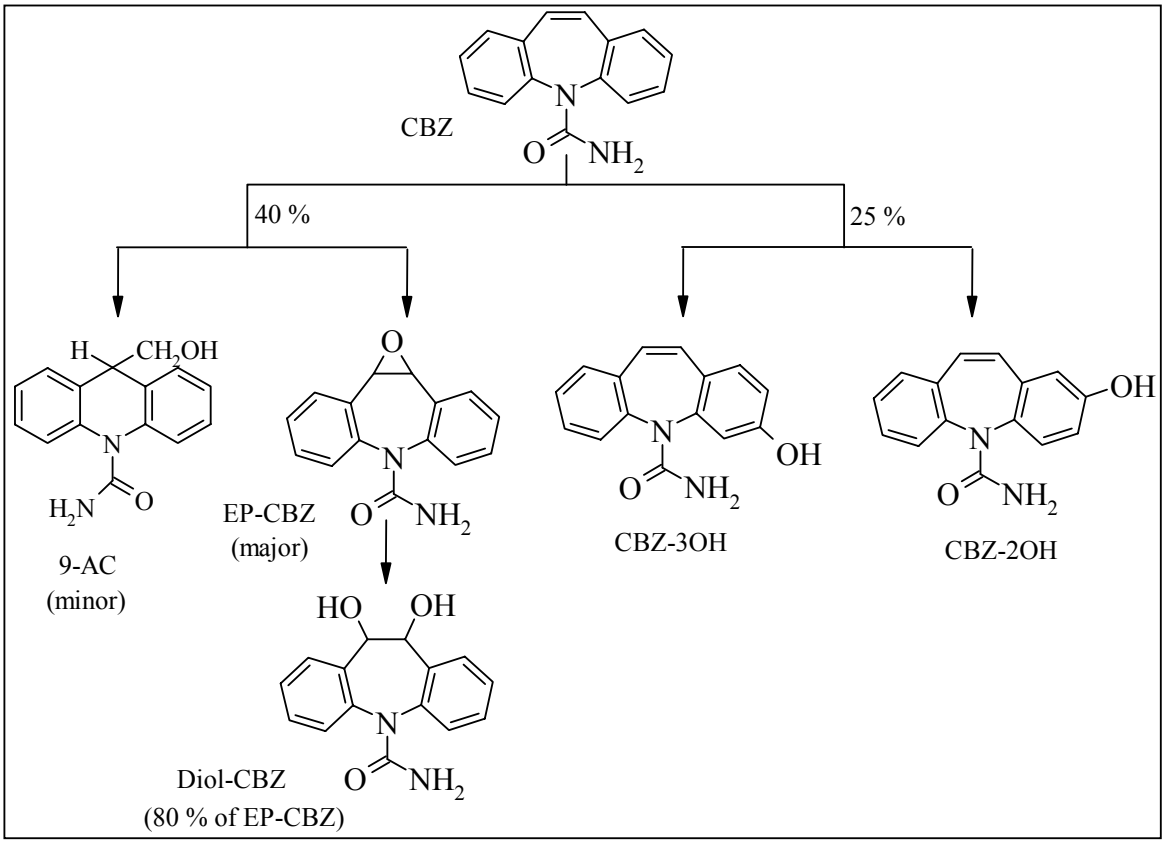


Fig. 2.1: Major pathways of the oxidative metabolism of CBZ in human [43]

DCF

The major metabolite from DCF in humans and rats is 4`-hydroxy-DCF (OH-DCF) (40%) [44]. Additionally, 10-20 % of each 3`-hydroxy-, 5-hydroxy-, and 4`,5-dihydroxy-DCF (Fig. 2.2) and 3`-hydroxy-4`-methoxy-DCF, furthermore an acyl glucuronide species was identified [45-46].

IBU

The metabolism of IBU in the human body is well known from pharmaco-kinetic studies.
Main excretion products including possible conjugates are IBU (15 %), OH-IBU (26 %), carboxyibuprofen (CX-IBU) (43 %) and carboxyhydratropic acid (CA-HA) in minor amounts [47-50] (Fig 2.3).

SFM

SFM is metabolised in the human body and about 50-60 % of the applied dose is excreted as the inactive metabolite Ac-SFM, 15 % as the conjugate Glucu-SFM and only 15-20 % as the unchanged compound [51-55] (Fig. 2.4).

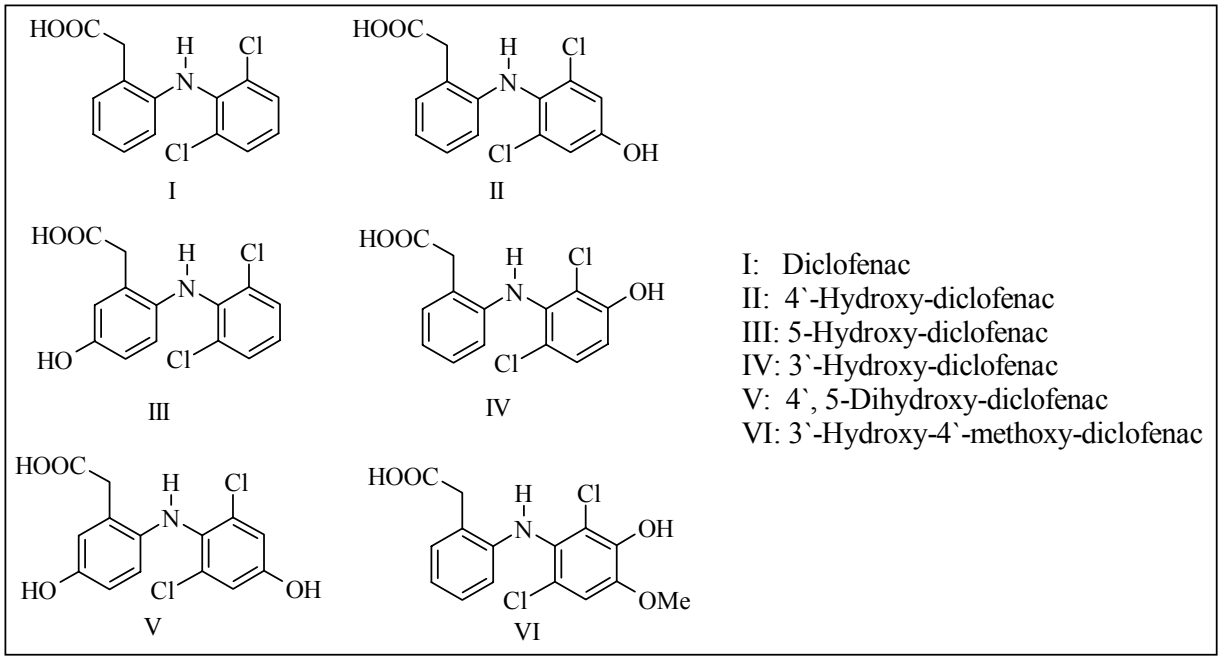


Fig. 2.2: The major oxidative metabolism products of DCF in urine [56]

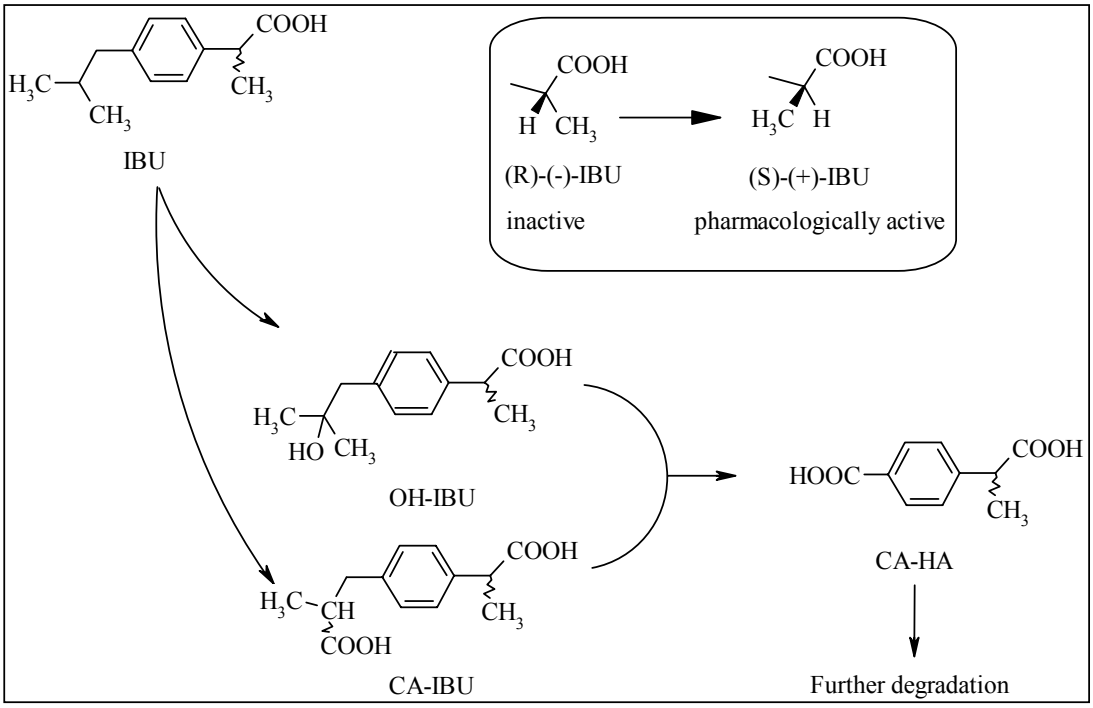


Fig. 2.3: Major pathways of the oxidative metabolism of IBU in human [57]

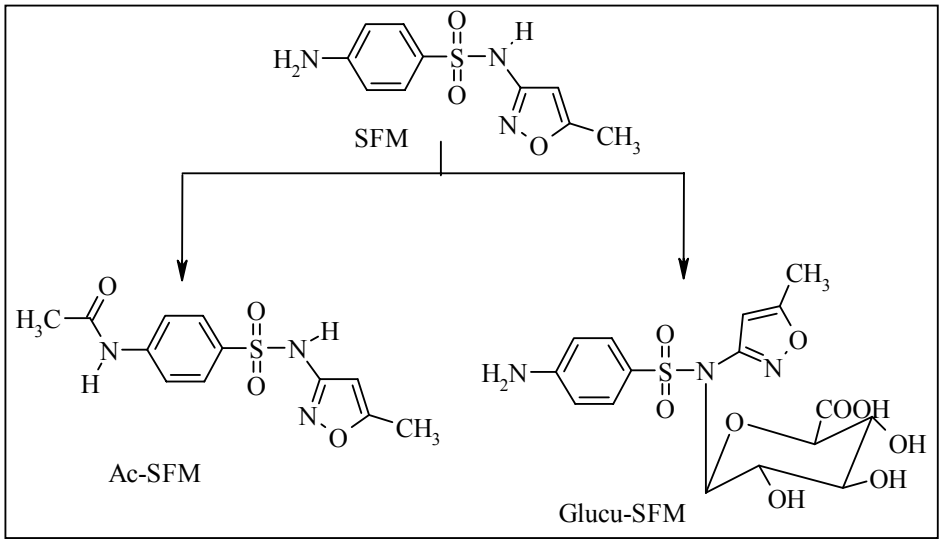


Fig. 2.4: Major pathways of the oxidative metabolism of SFM in human [58]

2.2. Fate of the drugs in aquatic environment

DCF

When DCF entering the water sources more than 90 % are degraded photolytically via formation of 8-chlorocarbazole-1-aceticacid (8-CCA) and carbazole-1-acetic acid (Fig. 2.5) [59-60]. The two compounds are not reported as human metabolites [61], however, they were previously identified as photolysis products of DCF in buffer solutions [62-63]. 8-CCA is considered to be the initial, cyclized dehydrochlorination product of DCF, and carbazole-1-acetic acid is a further photoreduction (i.e. dechlorination) product, in which chlorine is replaced by hydrogen. When the photolysis of DCF was carried out in pure water without a H-source (i.e. methanol), carbazole-1-acetic acid was not formed. Instead, another major product is formed, which was identified by mass spectra as hydroxycarbazole-1-acetic acid [60].

DCF showed in the laboratory experiment that there is only a negligible adsorption onto sediment particles and so it is not surprising that DCF was not detected in the sediments of lakes [60].

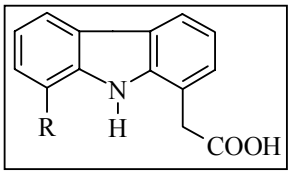


Fig. 2.5: The major DCF photodegradation products: (R: -H: carbazole-1-acetic acid, -Cl: 8-CCA, -OH: 8-hydroxycarbazole-1-acetic acid)

IBU

Under environmental conditions, this compound has different transformation kinetics.
Stumpf et al. [64] found that the excretion pattern is hardly changed on the way: Major changes occurred during biological treatment of the activated sludge in the STP and only slightly during primary pre-clarification. CX-IBU was almost quantitatively eliminated, while OH-IBU was the dominant compound in STP effluents and rivers.
This indicates that OH-IBU is the most stable of the three compounds under these conditions (if it is not continuously formed from IBU or from conjugate cleavage). Zwiener et al. [35] reported that OH-IBU was formed from IBU under aerobic conditions in activated sludge, while CA-HA was formed under anaerobic conditions. In both cases, these transformation products did not add up to more than 10 % of the initial IBU concentration, suggesting that the major amounts in sewage can be derived from human excretion.

For IBU, the direct phototransformation can be neglected because this compound does not absorb sunlight [65].

CBZ

CBZ is ubiquitously present in the aquatic environment [6]. However, the principle reason for the ubiquitous high CBZ concentration is the extremely low removal rate in municipal STP. Investigation of influent and effluent samples from different municipal STPs have shown that it is not significantly removed (less than 10 %) during sewage treatment [6]. Different field studies have shown that CBZ are not attenuated during bank infiltration [6]. This explains why CBZ has been detected in a number of groundwater samples at a maximum concentration up to 1.1 µg/L [6] and was also found with a concentration of 30 ng/L in drinking water [66]. However, as revealed by pharmacokinetic data only 1-2 % of CBZ is excreted unmetabolized. Glucuronide-conjugates can presumably be cleaved in sewage and STP and thus increase the environmental concentrations.

Because of its low biodegradability and chemical properties, photodecomposition becomes significant as a pathway of natural elimination. Despite the absorbance of sunlight by CBZ, Andreozzi et al. [67] observed only a slight elimination through phototransformation. On the other hand, Frimmel et al. observed that the presence of natural organic matter (HO19) increased the photochemical degradation rate of CBZ [30].

SFM

It was reported that SFM could be removed up to 60 % during the biological step in a municipal STP [68]. Heberer et al. [6] observed an efficient removal of various antibiotic and bacteriostatic drugs during bank filtration. In principal, SFM can be removed by traditional UV-irradiation as reported by Thiemann et al. [69].

There are only a few information available in literature about chemical behaviour and the fate of SFM in the aquatic environment [1].

3. Results and discussion

3.1. Methodical approach

This study is divided into two main parts:
In the first part pieces of animal intestines were tested as natural membrane for separation and enrichment of drugs from water. In the second part analytical methods were developed based on enrichment steps and GC/MS and/or LC-ESI/MS determination. Eventually, the methods should be applied to monitor the biodegradation of drugs in model plants.

Membrane study
Pieces of pig, sheep and cattle intestines were applied in a homemade permeation chamber device in order to investigate the basic membrane properties, such as membrane stability under various conditions and reproducibility of the drugs permeation. Furthermore, surface structure and pore size distribution were characterized. Moreover, direct influences on the permeation processes across the membrane wall like analyte concentration, surface area, stirring velocity, pH, temperature and the presence of other water ingredients such as inorganic salts, chelators, high molecular compounds and extracting materials are of great interest.
Additionally, the applicability of these natural membranes was tested with different aquatic matrices such as surface water, ground water, wastewater and sewage sludge water. Ultimately, comparative study between the applied natural flat membranes and technical membranes was carried out.
To fulfill this task at first the long-term stability of the selected analytes was to investigate and then LC/UV method was to develop for the registration of the permeated compounds.

Analytical method development
The second part of the present work will focus on the development and application of analytical methods for the simultaneous extraction and determination of acidic, neutral and basic target pharmaceuticals from environmental water samples. The specific approach will be done in the following steps:

- A pre-concentration step based on solid phase extraction applying different commercially available materials in order to find out the best material suitable for a wide polarity range of analytes

- Investigation in an optimal derivatization in order to render such compounds more volatility prior to GC/MS analysis

- Development of a GC/MS method based on SIM mode and assured by MS/MS for qualitative and quantitative analysis of the volatile analytes

- Development of a LC-ESI/MS method based on MS/MS technique for qualitative and quantitative analysis of the non-volatile and thermal labile compounds

- Testing the influence of water ingredients on the analytical methods (matrix effects)

- Validation of both methods by measuring the linearity range, repeatability, accuracy, limit of detection and limit of quantitation

- Examination of the developed methods for its intended purpose by analysing samples of surface, ground, wastewater and sewage sludge

- At least the biodegradation of the target pharmaceuticals and some of their synthesized metabolites will be followed for particular pilot plants such as batch and biofilm reactors model systems used in the Institute for Water Research in Schwerte-Geisecke

3. 2. Membrane Extraction

In this chapter the focus will turned to the possible applications of cheap animal intestine as natural membranes. The idea was based on such types of membrane used in the last century as dialysis membrane in medicine. So, the applying potential as membranes will be evaluated. The major concern in this work was given to some selected organic drugs as representative model.

3.2.1. Stability of the drugs stock solutions and monitoring of drug transport

As already mentioned, prior to the membrane studies, the stability of the aqueous drug solutions was investigated. Furthermore, HPLC-UV methods for the determination of the drugs and some of their metabolites were developed in order to follow permeation tests, both in feed and permeate side.

At the beginning of the study the stability of the single analytes in the aqueous stock solutions at different temperatures and pH-values were tested. For that purpose the aqueous stock solutions (2 mg/L) were stored in a water bath at (50 ºC) for 70 hours. After this treatment IBU, CBZ and SFM showed nearly no change in the concentration. Whereas, the concentration of DCF decreased about 20 %. Moreover, a new stock solutions were stored for 6 weeks in the refrigerator at 4 ^{o}C and afterwards further 6 weeks at room temperature at ~ 25 ^{o}C. CBZ, IBU and SFM showed no decomposition over a period of three months. In contrast, a loss of concentration of DCF was observed. In the refrigerator the decrease was about 5 % within one month, afterwards the concentration was constant until the bottles were stored at room temperature. From that time the concentration decreased continuously to 70 % (Fig. 3.1).
This loss in concentration could be attributed to a partly re-crystallisation of the analyte due to its low solubility and to the formation of oligomers (dimers up to hexamers) as has been detected by LC-ESI/MS. It is known that in some cases the source of electrospray can play an important role in adduct formation [107]. Many attempts were made to separate those oligomers by liquid chromatography to confirm oligomers formation in solution. Oligomeres could not be detected in a fresh solution of the same concentration. In our case, the oligomerization observed might be an effect of aging favoured by higher concentrations. Therefore, the concentration of the stock solutions should not be higher than 25 mg/L. Also, they should be stored in the refrigerator in order to decrease the degradation processes. It is recommended to treat the solutions with ultrasonic at a bath temperature of 40 ºC for 15 minutes before carrying out membrane studies.

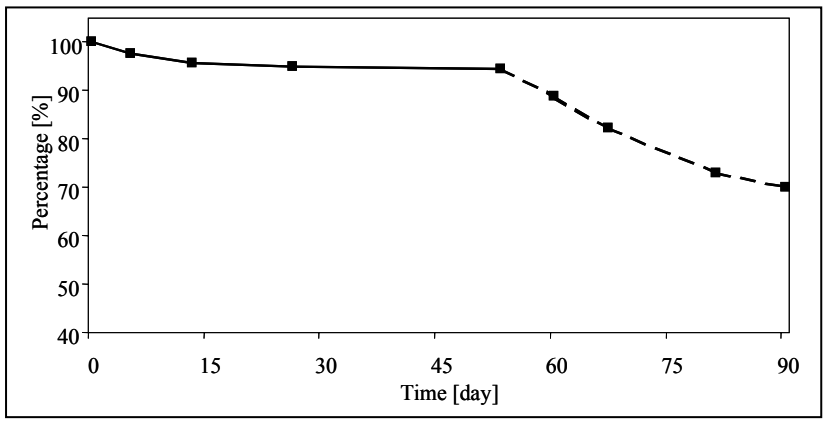


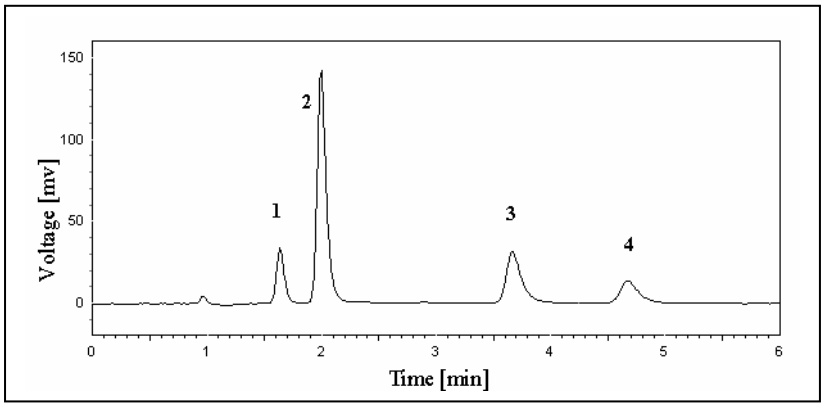
Fig. 3.1: DCF stability in water as function of time and temperature (10 mg/L); 0-43 days: 4 ºC, 44-90 days: 25 ºC

Development of HPLC-UV methods

The transport of the analytes was monitored by HPLC and UV-detection at a wavelength of 225 nm. Aliquots were taken from the liquid phases at intervals by means of a micro-litre syringe. The developed methods for the selected pharmaceuticals and some of their metabolites are described as shown in the chromatogram (Fig 3.2) and described in section 5.3 (Method I+II). The external calibration curves were built from 6 concentrations (n=3) in a concentration range of 0.1 - 5 mg/L. In the membrane tests the starting concentration of the drugs were 10 mg/L and the pH-value was adjusted to 9.5 at the feed side. The permeation phase was deionized water and the solutions in both chambers were stirred (500 RPM) in order to avoid a concentration gradient within the chambers.

The different pH values (3 and 10) did not influence the substances SFM and CBZ. In the other hand, pH 3 had nearly double the response of DCF and IBU as well. Therefore, the calibration standards should be at the same sample pH level in order to compensate the higher response.

A)



B)

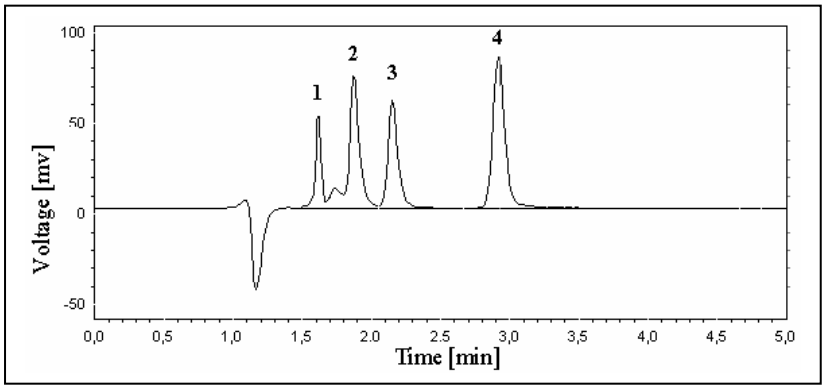


Fig. 3.2: HPLC-UV chromatogram for the selected pharmaceuticals and some of their synthesized metabolites, **(A**: 1. SFM, 2. CBZ, 3. DCF, 4. IBU, **B**: 1. Glucu-SFM, 2. Diol-CBZ, 3. OH-IBU, 4. Ac-SFM**)**, Nucleosil-120 C-18 as analytical column, acetonitrile/NaH_2PO_4-buffer mobile phase (225 nm)

3.2.2. Natural flat membranes

History

Animal intestines are available on the market and used since ancient times in food industries as packaging material [70]. Moreover, they were used as membrane for dialysis in the last century to discharge the blood from toxic substances [71].

The principle has been used to design highly efficient artificial kidneys based on dialysis [102]. In this process the blood from a patient suffering from acute or chronic kidney failure is passed into dialyser from a connection to one of his arteries. Low molecular weight toxins in the blood, such as urea, creatinine and uric acid pass a cross the membrane into a dialysate solution of such composition that the osmotic pressure is the same as that of the blood; the rate of transport of certain salts is thus controlled. The blood then returns to the patient through a connection to one of his veins.

Transport mechanism

The oral absorption of a drug administered in a solid dosage form depends on a series of events which occurs in the intestinal lumen: the release of the drug from the solid dosage form; dissolution of the drug in the luminal fluids; drug metabolism and transport of the drug molecules from intestinal lumen to the blood across the intestinal wall. The rate-limiting step in the absorption of many orally administered drugs is the transport across the intestinal wall.
The small intestinal wall consists of three main layers: the muscularies mucosae, the lamina propria, and the epithelium [72]. The rate limiting barrier is the intestinal epithelium. In i*n vivo* systems, drugs are usually transported across the intestinal epithelium by one or more of the following routes: 1. the passive transcellular route; 2. the passive paracellular route; 3. active carrier-mediated transcellular routes; 4. the transcytosis route (Fig.3.3) [19].

Most of the transport occur by the transcellular route since the membrane has a surface area which is over 1000 times greater than the area of the paracellular spaces [73]. Several molecular properties, such as size, charge, lipophilicity and conformation, could influence the passive transcellular transport of drugs across intestinal epithelial cells [74].

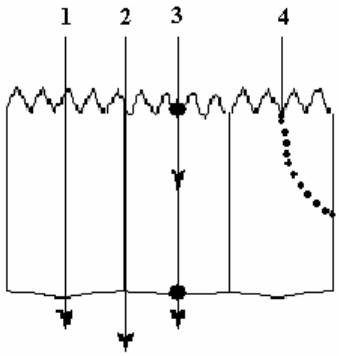


Fig. 3.3: Drug absorption routes across the intestinal epithelium [19]

Selection background

The transport mechanism through the natural cell wall of the intestines of living animals could happen in different routes described above, in contrast the separation process using technical membranes are mainly a function of molecular size and pore size distribution.

The reasons for the investigation to apply natural organic material are based on the splendid permeation characteristics and the high enrichment factor achieved with living cells in medicine. Our experiments should reveal, if similar transport properties would be maintained under in vitro conditions.

Origin, preparation and enrichment device used

Pieces of animal intestines such as from cattle, sheep, and pig were used as membranes.
Fig. 3.4 shows the technical device constructed to test the natural flat-membranes. The membranes were fixed in a window between the feed and permeation PTFE-chamber with

silicon seals. Both chambers were closed with caps to avoid loss of water by evaporation and the solutions were stirred on both sides by micro magnetic stirrers. Different setups have been modulated with two different sizes of 150 and 450 mL. The chamber volume was filled up to 4/5 of the nominal volume.
Before the special part of the cattle appendix, called 'Goldschlägerhäutchen', was finally selected for the further tests, other parts of the intestines of cattle, sheep, and pig had been studied, as example pig and cattle intestines (Fig 3.5). They showed similar permeation trends, however, Goldschlägerhäutchen was favourable because large pieces are available in different dry and wet form and faster equilibration time is remarkable.

Goldschlägerhäutchen, normally used as sausage skins, are available dried or wet under preservation of salt. For membrane application, the salted product has to be watered in order to get rid of the salt. Afterwards, it can easily be inserted between the silicon seals of the membrane device though its surface is a little slippery. The fate layers were already separated from the delivered intestines. The dried trading products have a surface of about 30 x 40 cm, the wet ones of about 50 x 70 cm.

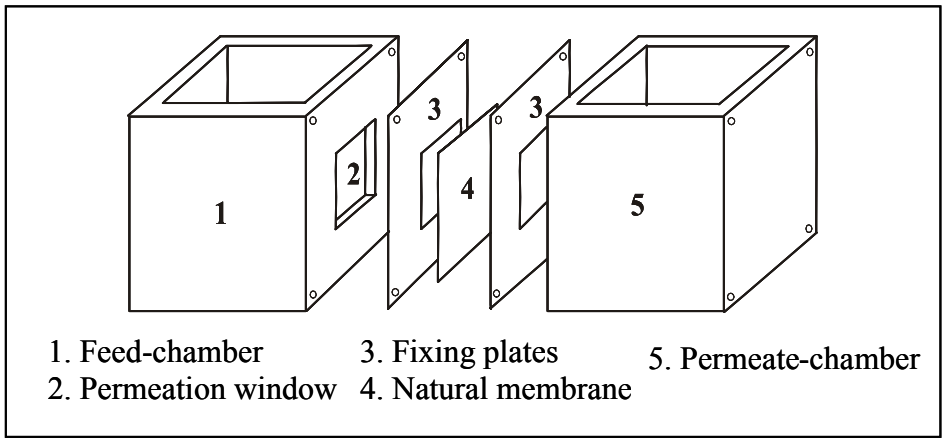


Fig. 3.4: Technical device used for natural flat membrane tests

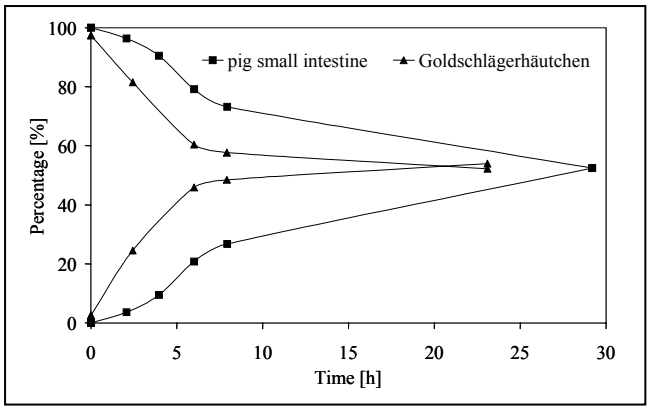


Fig. 3.5: Comparison of drugs permeation between Goldschlägerhäutchen and small intestine of pig (15.2 cm^2; membrane area); 10 mg/L of each pharmaceutical

3.2.3. Natural flat membrane characteristics

3.2.3.1. Membrane stability

In order to have knowledge how long this membrane remains working under various conditions, we tested pieces of the membranes by soaking them up to three months in NaOH solution (pH 10) and HCl (pH 2). These values were selected as extreme values because in the normal wastewater the pH-value ranges usually between 8 and 9 and because in water analysis natural water samples are usually acidified to pH 2. The membranes were stable at pH 9, whereas, in the dilute acid they were destroyed within 3 weeks.
Then these stressed pieces were compared with fresh wet and dry pieces. As shown in Fig. 3.6 there were no significant differences among the different membranes.

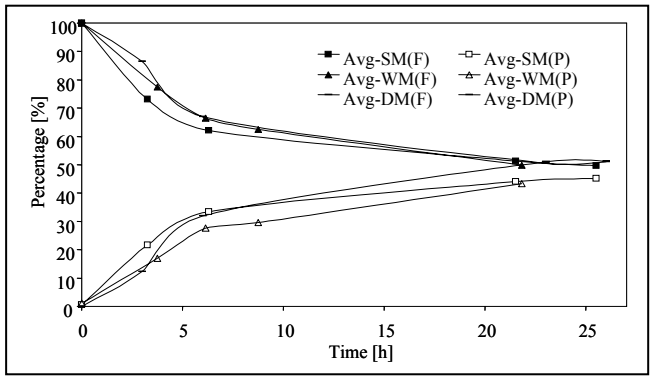


Fig. 3.6: Comparison of pH-stressed membranes with fresh wet and dried membranes, SM = 3 months in basic solution, WM = new wet membrane, DM = new dried membrane, membrane surface area (8.1 cm^2), F: Feed, P: Permeate, Avg: the mean from the four drugs

We found different membrane stability based on the water source. In ground water the membranes were stable for more than two weeks. Even in sewage sludge water we observed similar stabilities. Wastewater and wastewater effected surface water destroyed the membranes within 1-2 days because of their high bioactivities. By adjusting the water to pH ≈ 2 the stability can be extended to more one day.

There are two possibilities to overcome this problem: The application of photolysis or chemical cross-linking. The water matrix was photolysed for different periods of time to reduce any present bioactivity by using UV-lamp radiation. But this operation elongated the stability only for one further day. A second possibility of stabilisation is based on cross-linking with formaldehyde [75]. For this purpose the membrane was placed in a formaldehyde solution (1 %) for two hours, then washed and dried. Such prepared membranes could extend their stability up to 10 days.

3.2.3.2. Reproducibility of permeation properties

The reproducibility of the membranes permeation process was tested by series of tests under the same conditions. After the first run the same membrane piece was used repeatedly with fresh feed solutions three times. Then the membrane position was changed in such a way that the surface which contacted at first the feed side was turned to the permeation phase in a second series of measurements. The results are presented in (Table 3.1). Furthermore, different pieces of one intestine batch were investigated under the same conditions. It is

remarkable that the permeation characteristics are nearly independent on different batches as well of the position of the surface.

Table 3.1: Reproducibility for piece of membrane on both sides

	Time [h]	F [%]	SD [%]	P [%]	SD [%]
M-Fr	3	80.0	*3.2*	17.3	*1.5*
	6	72.1	*2.1*	30.9	*1.7*
	24	51.4	*3.9*	46.4	*1.4*
	27	48.3	*2.7*	47.9	*1.8*
M-B	3	78.5	*6.8*	14.2	*3.1*
	6	67.0	*6.7*	27.8	*4.3*
	24	49.5	*3.5*	45.0	*3.2*
	27	49.3	*3.7*	45.9	*3.1*

M = membrane, Fr = front side, B = back side (by definition); n = 4
F = feed side, P = permeate side, SD = Standard deviation

3.2.3.3. Membrane surface structure and pore size

Scanning electron microscope studies have been done to get an impression about the structure of the intestine surfaces (Fig. 3.7). For all wet membranes we observed a somewhat cloudy structure with little remarkable details. Against that, the surface of the dried pieces showed a relative strong structuring, resulting from the protein conglutination of different individual parts. In Fig. 3.7 a pore structure is not obvious.

Because of the effect that about 10 % of a standard humic substance (HUS; HO13), having a molecular weight > 1000 Dalton, permeated through the membrane within the first 24 hours, an attempt was started using polyethylene glycol (PEG) with a molecular weight of 1500 and 3500 in order to characterize the pore size. The PEGs needed about 20 hours to reach equilibrium on both sides (Fig. 3.8). PEG tests were analysed by HPLC and refractive index detection (RI) as described in section 5.3 (Method III). While, HUS test was measured by spectrophotometer at 254 nm.

However, no clear information of pore size distribution can be given by these results. Presumably, the test substances transport through the membrane wall by diffusion as described in section 3.2.2. Therefore, the question of correct pore size could not be answered exactly.

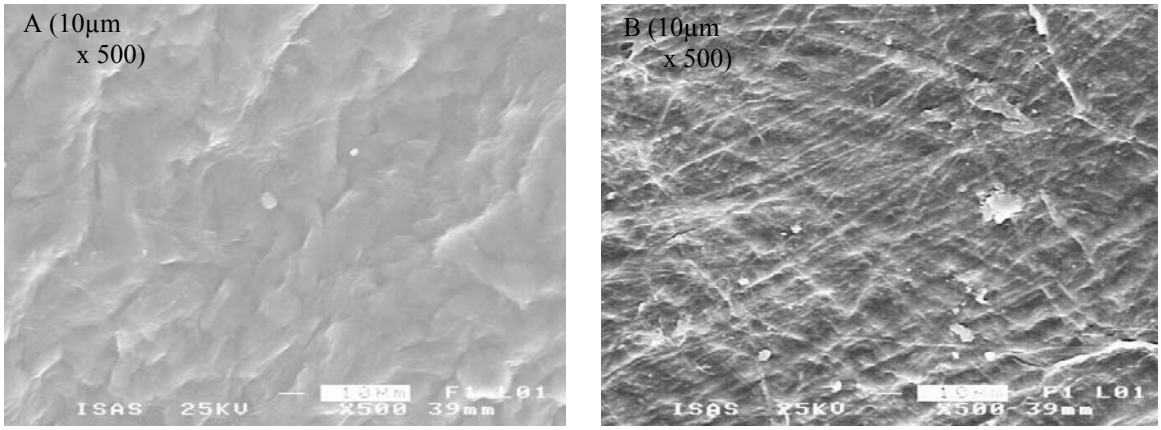


Fig. 3.7: Scan electron microscope of the Goldschlägerhäutchen (A) and Sheep-Appendix (B)

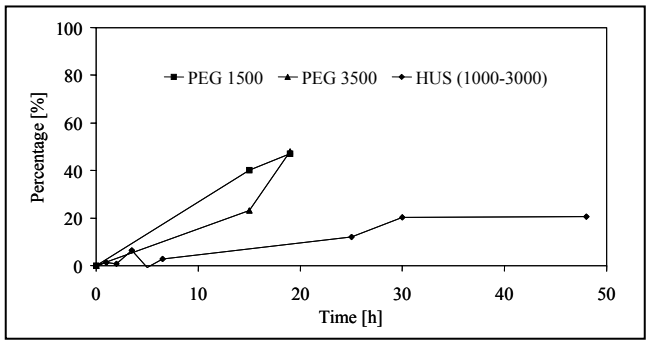


Fig. 3.8: Goldschlägerhäutchen pore size characterization using 1 % PEG, 10 mg/L HUS (HO13) and membrane surface area (8.1cm^2), HUS was measured at 254 nm and PEG using HPLC/RI (Fractogel TSK HW-40S was used as stationary phase and water as mobile phase)

3.2.4. Influences on permeation characteristics

3.2.4.1. Influence of general parameters on the permeation process

The influence of some basic parameters such as membrane size (Fig. 3.9), stirring speed (Fig. 3.10) and temperature (Fig. 3.11) on the permeation of SFM, CBZ, DCF and IBU were studied in addition to some of their synthesised metabolites; Diol-CBZ, OH-IBU, Ac-SFM and Glucu-SFM.. The optimum settings were kept for all succeeding tests. Though the selected compounds differ in structure and polarity, they represent always the same permeation behaviour as demonstrated by the result in (Fig. 3.14). Moreover, the synthetic metabolites showed similar trends of permeation. Therefore, the drugs permeation results were demonstrated by the average values from the all test analytes as one unit.

As expected, the relationship of chamber volume and membrane size was important and although the temperature influenced the dynamic of the permeation, the stirring velocity was of particular importance. When increasing the velocity a decrease of the equilibrium time was observed. A further increase above an optimal value resulted in turbulent flows without further enhancement. A velocity of 500 rotations per minute was fixed as the practical optimum.
The influence of different drug concentrations on the permeation process as presented in (Fig. 3.12) is low.

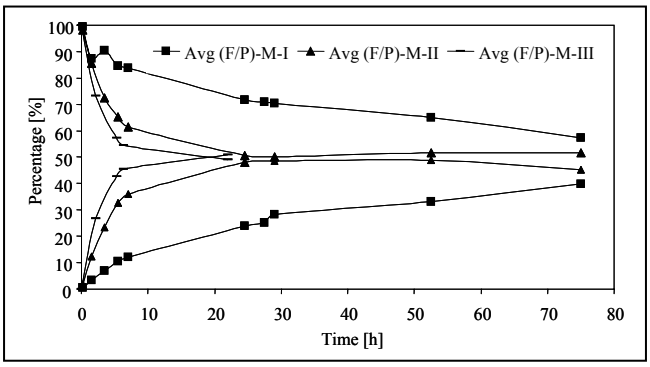


Fig. 3.9: Influence of membrane surface area in cm^2: M-I 3.84, M-II 15.2, M-III 25

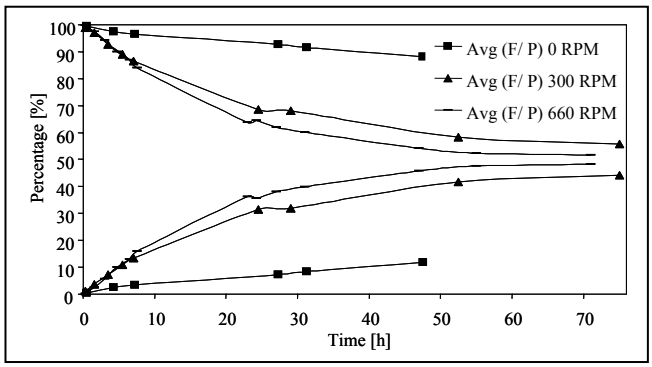


Fig. 3.10: Influence of stirring rate (3.84 cm^2)

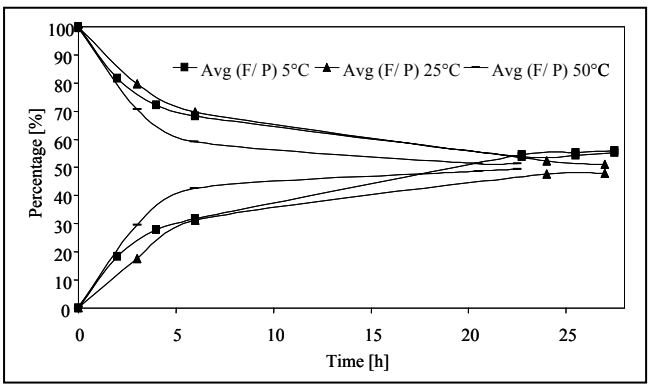


Fig. 3.11: Influence of temperature (15.2 cm^2)

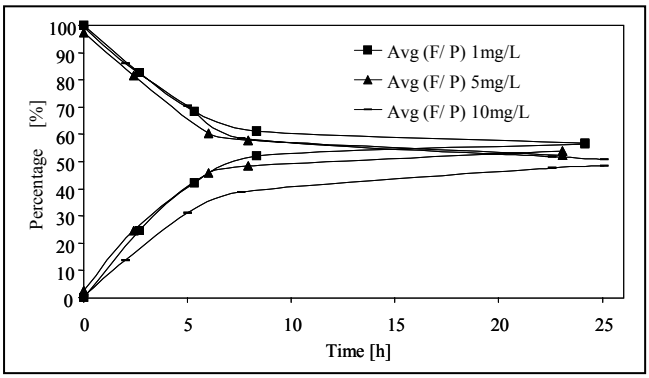


Fig. 3.12: Influence of concentration (15.2 cm^2)

No significant influence of pH on the analyte transport through the membrane could be observed (Fig. 3.13). The reason might be found in the pH-balance between both chambers, which is achieved within a very short time. Throughout the most experiments the feed samples were adjusted within pH 8 - 10, representing the pH range of wastewater.

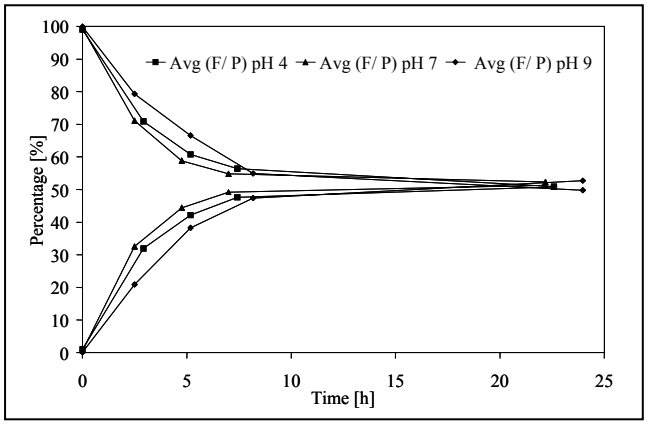


Fig. 3.13: Influence of pH (15.2 cm^2)

3.2.4.2. Influence of additives on the permeation process

To increase or hinder the permeation process many additives were added to the feed and permeate solutions such as salts, chelating agents (β-CD, EDTA) and surfactants (ionic and non-ionic).

The results we got from the addition of salt (Fig. 3.14) and chelating agents (Fig. 3.15-16) referred to no actual variation in permeation kinetics.

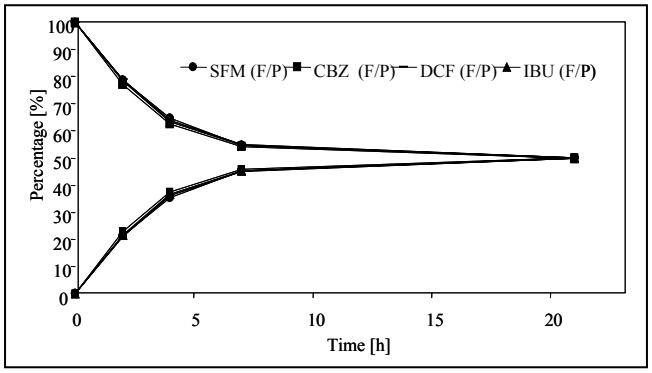


Fig. 3.14: Influence of salt; 1% (w/w) Na_2SO_4 (15.2 cm^2)

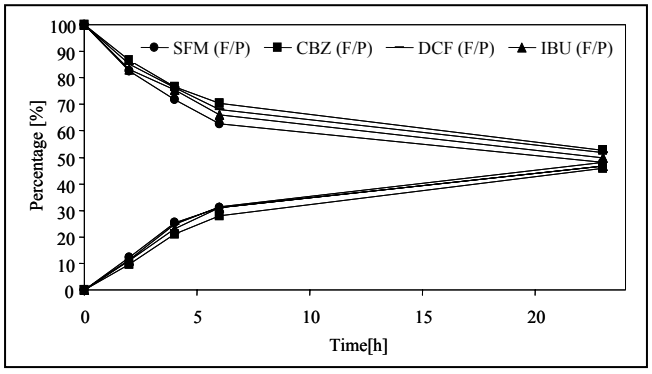


Fig. 3.15: Influence of chelators; 0.1 % (w/w) EDTA+ 1 % (w/w) Na_2SO_4 (15.2 cm^2)

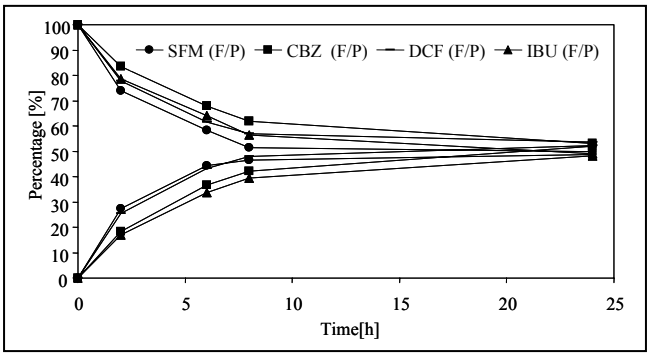


Fig. 3.16: Influence of chelators; 0.5 % (w/w) β-CD (15.2 cm^2)

In the presence of other ingredients, e.g. by addition of sodium dodecylsulfate (SDS; ionic) or polysorbate (non-ionic), the analyte transport was affected adversely (Fig. 3.17-18).
But that depends on the concentration of the surface-active or complex-forming agent; when the concentration of the extra is > 0.5 % a tangible retardation is the consequence for CBZ and DCF. However, in the concentration range known to be present in wastewaters the effect can be neglected and, besides that, it can be attenuated by relative high salt concentrations (salting-out effect).

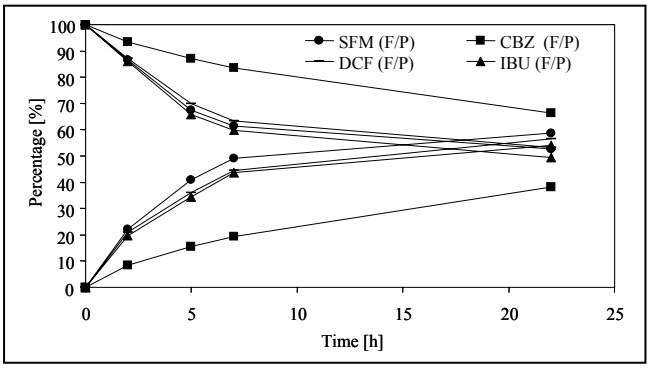


Fig. 3.17: Influence of tenside: 2 % (w/w) SDS added to feed side (15.2 cm^2)

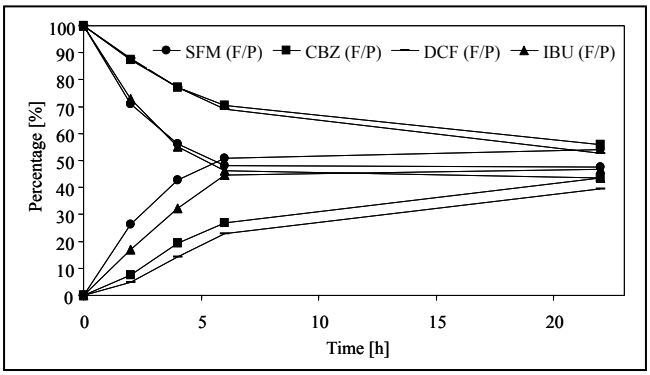


Fig. 3.18: Influence of complexation agent: 1 % (v/v) polysorbate added to feed side (15.2 cm^2)

3.2.4.3. Influence of aqueous matrix on the permeation process

Wastewater contains a high spectrum of substances like salts, HUS and other organic and inorganic compounds. Therefore, we have investigated the behaviour of the drugs in the presence of HUS (Fig. 3.19). Two HUS fractions were added in different batches; it was the standard material Hohlohsee13 (HO13, Black Forest area) and Venner Moor (VM, North-Rhine-Westphalia district).

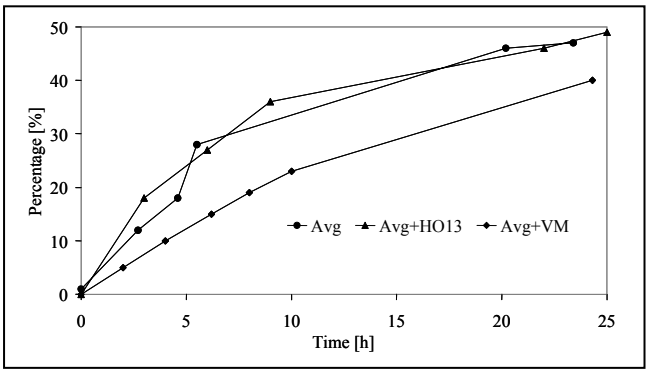


Fig. 3.19: The influence of the 10 mg/L hydro-colloids HO13 and VM on pharmaceutical permeation through the natural membrane (8.1 cm^2), Avg = average values

Because of their chemical composition the presence of such high molecular weight hydro-colloids like HUS can lead to different interactions with the analytes such as hydrogen binding to polar groups, or covalent binding, or only coordination. This again can have a direct influence on analyte permeation kinetics. Actually this is the fact for HUS of a younger genesis such it is the case of bog-water from the Venner Moor Fig. 3.19. The lower curve in the Fig. 3.19 is caused by analytes with polar structure moieties as the carboxylic groups of DCF and IBU. But in the same figure it is also demonstrated that no significant influence was observed in the presence of the HUS (HO13), which had reached its final stage of humification.

Other investigations have been done using real water samples resulting from sludge and wastewater (Fig. 3.20-21). It couldn't be found any influence on permeation by filtrated sludge and wastewaters.

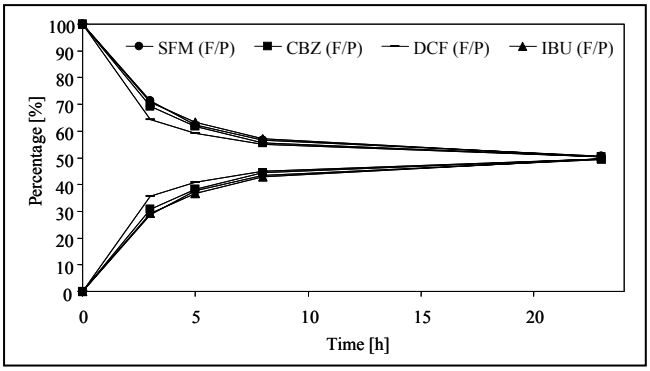


Fig. 3.20: Influence of the filtrated sludge water on the permeation (15.2 cm^2)

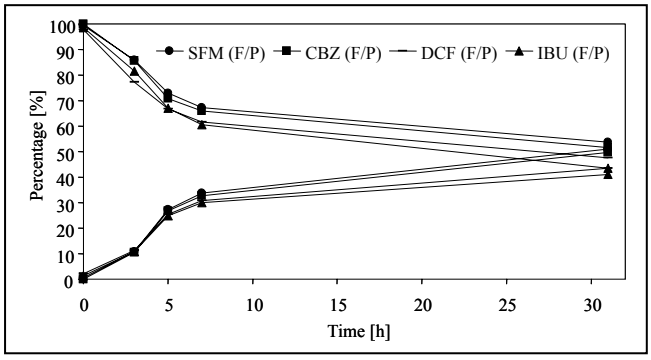


Fig. 3.21: Influence of the filtrated wastewater (pH = 3.0) on the permeation (15.2 cm^2)

3.2.4.4. Influence of additional extraction steps on the permeation process

It is characteristic for permeation processes in a defined volume that only an equilibrium concentration can be reached between both sides. To improve the recovery, the concentration gradient must be forced. This can be achieved by removing the analytes from the permeate chamber using an additional acceptor phase. The target pharmaceuticals could be extract from the permeate phase as reported by M. Grote et al. [105] by overlaying with water immiscible liquid phase. The concept of liquid/liquid extraction was carried out by overlaying the permeate phase a water-immiscible film of an organic solvent such as n-octanol or a mixture of n-octanol or n-decanol with n-decan in different ratios (Fig. 3.22-23). Furthermore, we tested the influence of additives on the extraction such as tris(2-ethylhexyl)-phosphate in order to enhance the selectivity and the efficiency of the extraction processes (Fig. 3.24).

In the system with octanol CBZ was eliminated with 80 %, DCF and IBU with 60 % from the feed side, while SFM showed no improvement. The system with the solvent mixture increased the depletion for CBZ of only about 10 %. Additives did not show any remarkable improvement on the permeation efficiency.

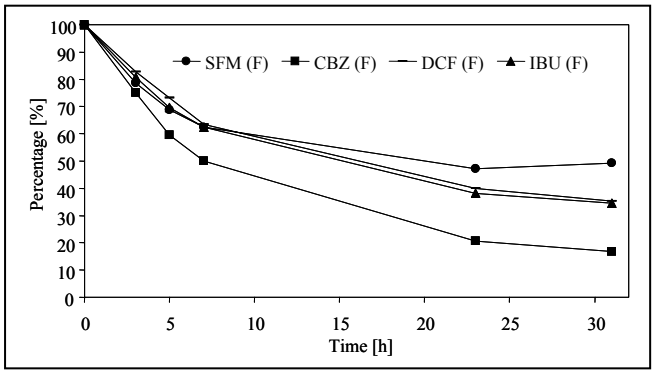


Fig. 3.22: Influence of an additional liquid phase extraction on the permeation using n-octanol (15.2 cm^2)

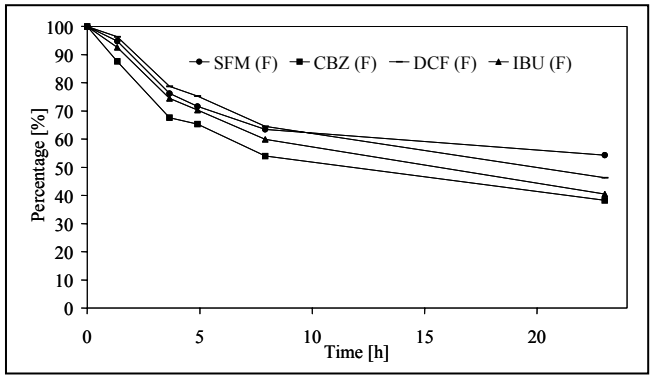


Fig. 3.23: Influence of an additional liquid phase extraction on the permeation using n-decan: n-decanol (80:20) (15.2 cm^2)

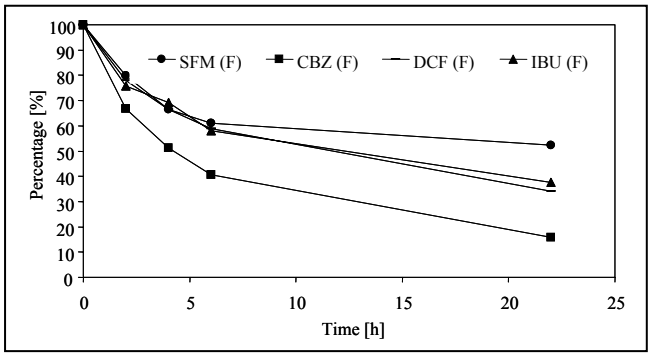


Fig. 3.24: Influence of an additional liquid phase extraction on the permeation using n-octanol in addition to 2 ml tris(2-ethylhexyl)-phosphate (15.2 cm^2)

Another way for the depletion is an extraction by means of solid adsorbent materials. The most effective procedure is pumping of the permeation solution through a column filled with charcoal as effective adsorbent materials (Fig. 3.25). The result showed more than 90 % of all test compounds could be eliminated from the feed chamber. An immediate use of charcoal filtration without the membrane technique is inappropriate because a clogging of the filters by particles and high molecular compounds would deteriorate the sorption efficiency. Hence, the natural membrane has an important role as retardation reactor to prevent the adsorbent material from the overloaded processes.

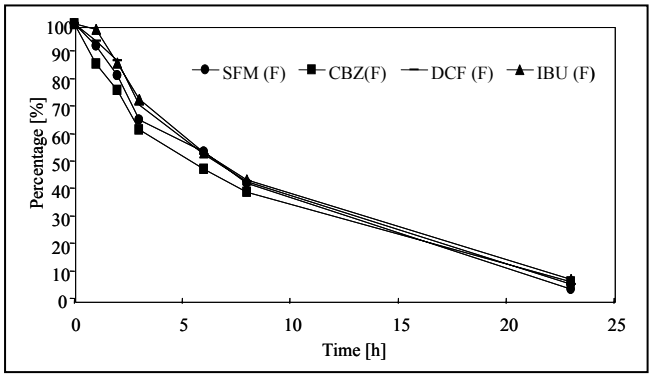


Fig. 3.25: Influence of an additional solid phase extraction on the permeation using charcoal column (15.2 cm^2)

3.2.5. Comparison between natural flat membranes and technical membranes

In order to get an idea where the natural membranes are positioned in comparison with technical membranes used in dialysis, cellulose ester membranes with a molecular weight cut off of 1000 Dalton has been tested. An additional question was how they behave in the presence of HUS of younger genesis (VM)(Fig. 3.26).

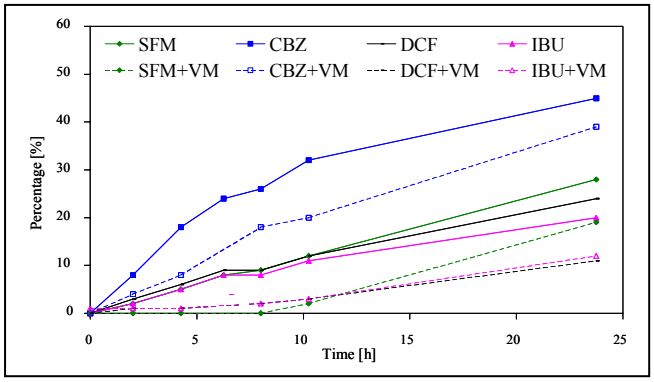


Fig. 3.26: Influence of humic substances on the permeation of pharmaceuticals, using a technical membrane (Cellulose ester; MWCO =1000 Dalton) (8,1 cm^2)

In the Fig. 4.26 it can be noticed that CBZ permeated similarly to the natural membrane but the other pharmaceuticals permeate retard clearly. While the permeation yield of CBZ was about 45 %, the one of the other substances were less than 25 %. This loss increased especially in the presence of HUS, which had an effect to all substances of about 10 %.

Another interesting point, demonstrated in Fig. 4.26, is that CBZ permeated relative quickly within the first 8 h in comparison to SFM, DCF, and IBU. During that time SFM seems to have no permeation but afterwards the kinetic increased clearly stronger than IBU and DCF. The influence of the HUS besides the above mentioned interaction with the analytes may be caused by adsorption onto the membrane surface or a blocking of the pores by HUS/analyte-adducts [104].

3.2.6. Conclusion

As shown by the single results presented in this chapter, it is obviously that intestines from animals can work as dialysis membrane. Different types of intestine parts of cattle, sheep and pig were applied in the original wet and dried form. The permeation is mainly influenced by the concentration gradient between the both chambers. Therefore, increasing the stirring velocity and membrane surface area to optimum leads to a decrease of the equilibrium time to about six hours. pH values, temperature and concentration in both champers showed nearly no influence in the total permeation process.

Goldschlägerhäutchen was chosen for further investigations because large pieces are available in different dry and wet form and faster equilibration time is tangible.

The interferences with water ingredients such as chelating agent and surfactants can interfere the permeation process but only in unusual high concentrations. Additional water matrices such as humic substances could retard the permeation process of some analytes depending on their chemical structure and the composition of the matrix as well. To increase the depletion of the analytes a re-extraction by solid or liquid phases must be performed. Activated carbon showed to be a superior combination method. A depletion of more than 90 % of all analytes is feasible.

The behaviour of the studied analytes and in dependence on the test conditions was investigated with stocks solutions. Within three months, all stocks solutions showed remarkable high stabilities except DCF, which had a loss of about 30 % from the original concentration. The loss could be interpretated to a partly re-crystallization or photodegradation as well. Therefore, the stock solutions should not be higher than 25 mg/L and should be store in the refrigerator.

In such samples the natural membranes showed high stabilities and reproducible results. But the stability is decreased tremendously in presence of a bioactivity in the water samples. In the presence of bioactivity the lifetime of the membranes are not satisfactorily. To overcome that problem and to increase the lifetime for some more days, the bioactivity should be decreased by pre-filtration or radiation with UV-light and the membranes should be treated with formaldehyde solutions.

Different polyethylene glycols and standard fractions of humic substances were applied to estimate the pore sizes. No clear answer of the pore size could be given because the permeation through the membrane presumably occur by diffusion as described in theory of the membrane in vivo system. Then the intestines were compared to technical membrane. In the presence of humic substances some of the analytes were highly retarded from cellulose ester dialysis membrane with fixed molecular weight cut off of 1000 Dalton. This result revealed that the natural membrane used miscellaneous pathways in permeation processes.

As a consequence, possible applications in water treatment are restricted by membrane stability. Furthermore, analytical enrichment is not postulated due to slow permeation kinetics and a second extraction step is necessary after membrane permeation for the permeate side.

3.3. Methods development based on LC/MS and GC/MS

Besides improved separation and enrichment procedures reliable analytical techniques based on an enrichment step, chromatographic separation and mass spectrometric determination with and without derivatization are fundamental. Therefore, in this chapter the attention is turned to it in the following units.

Though, advantageously in water treatment natural flat membranes are unsuitable for analysis purpose for two main reasons as point out in previous conclusion: the first, the permeation velocity is too slow, hence the total time analysis would be extended unfavorably. The second, a second extraction step is necessary after membrane permeation for the permeate side. If necessary, a filtration with conventional filter is without problems, because only maximal sample volume of 1 L is needed.

3.3.1. Real water sample preparation

Sample clean up and extraction
In order to remove suspended material, the aqueous samples were vacuum filtered through 0.45µm cellulose membrane filter.

One intention of this work was to develop a method for the extraction of acidic, hydrophilic, neutral and basic pharmaceuticals simultaneously from water samples. Generally, a selective extraction of pharmaceutical compounds from environmental waters is a critical step but in this study it was especially difficult, because the analytes cover a broad range of polarity. Ultimately, the final enrichment method could only be the best compromise.

As pointed out in chapter 1, for the extraction of high polar analytes SPE with polymeric sorbents often proved to be superior to alkyl-bonded silica (e.g. octadecasilane). A variety of hyper-crosslinked sorbents are commercially available, differing in the degree of linkage, porosity and surface area. They are either co-polymerisates of styrene and a polar component (e.g., methacrylate or *N*-vinylpyrrolidone) or the functional groups are introduced after polymerisation (e.g., by sulfonation). This functionalisation results in mainly two effects: improved wetting characteristics for better mass transfer and additional possibilities for interactions with functional groups of the analytes and thus a higher degree of retention.

At the preliminary stage in this study, a variety of cartridges were investigated such as octadecasilane (C-18), ethylvinylbenzene-di-vinylbenzene-copolymer (Lichrolut EN) and [poly(divinylbenzene-co-N-vinylpyrrolidone)] (Oasis HLB).

To avoid contamination of plasticizers which may be caused by the polypropylene martial, glass cartridges were used exclusively. These cartridges were self-filled with the SPE materials. The amounts of filling were listed in Table 3.2. The influence of the adsorbent amount was investigated successively by the recovery. The yields were calculated from 1 L of ultrapure water (pH ≈ 2) spiked with 1 µg/L each of the analytes by using a HPLC/UV method which allowed the separation and quantitation of all selected analytes as described in section 3.2.1. The results are listed in Table 3.2.

Oasis HLB adsorbent was finally chosen as the best phase for our purpose. The structures of the compounds studied and the pk_a values of the investigated drugs are shown in Table 1.1.

The samples were acidified to pH 2 as water samples are normally stabilised at that pH to obtain the undissociated form of the analytes. Several parameters especially the elution solvent, the drying temperature and time were optimised for conditioning the SPE material. The following conditions were fixed as represented in Fig. 3.27 and described in the experimental section as well.

To achieve sufficient sensitivity, or to change the solvent for further analysis, the extracts had to be concentrated by evaporation of the solvent, occasionally several times within the complete analytical procedure. The volume reduction could be critical and results in losses of the more volatile compounds, especially of IBU, had to be considered. Therefore, special care was taken and the sample was not allowed to come to complete dryness.

Table 3.2: Percentage of drug recoveries and relative standard deviation (RSD) obtained with various SPE cartridges (concentration: 1 µg/L, 1 L; pH = 2 and n = 3), (LC-UV)

Sorbent	C-18 (500 mg)		Lichrolut EN (200 mg)		Oasis HLB (100mg)	
Analyte	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
SFM	22	22	45	7	71	4
CBZ	86	16	97	0	94	0
DCF	98	14	80	4	100	4
IBU	85	3	83	3	95	2

Derivatization for GC-MS

The major purposes of analytical derivatization are the enhancement of the volatility of analytes by decreasing the polarity, for example of OH-, COOH-, NH_2- groups or increasing the thermal stability. In order to render such compounds more volatile prior to GC analysis, they are mostly esterified, silanated or acetylated using one of the diverse methods reported.

The selected pharmaceuticals in this study are characterised by a wide polarity range; IBU has an acidic group, SFM, DCF are characterised by both acidic and basic groups in the same molecule and for CBZ the basic group is dominant.

The best choice for derivatization of acidic pharmaceuticals containing carboxylic moieties is given by methylation with trimethylsulphonium hydroxide (TMSH) or diazomethane (CH_2N_2). Other possibilities besides methylation are acylation with trifluoroacetic anhydride (TFAA), benzylation with pentafluorobenzyl bromide (PFBB) and silylation with N,O-bis(trimethylsilyl)acetamide (BSA) [91]. CBZ contains an amide and SFM a sulfonamide and a primary amine as functional groups which may be derivatised by alkylation, silylation or acylation reagents depending on their special structure.

Based on qualitative experiments, many reagents from divers described methods were investigated for analytical derivatization concerning the functional groups of the analytes, namely esterification [34,85-86,91], alkylation [82,87], silylation [88-89,103] and acylation [84]. The qualitative results could be illustrated as the following:

1. Methylation with CH_2N_2: suitable for DCF, IBU, OH-IBU, SFM and Ac-SFM (Fig. 3.28)

2. Benzylation with PFBB: suitable for DCF, IBU, and SFM (Fig. 3.29)

3. Silylation with BSA: suitable for DCF and IBU (Fig. 3.30). CBZ could be silylated by MSTFA/TMSI/ DTE 1000:2:2 (v/v/w) only in very low yields (Fig. 3.30c)

4. Acylation: different reagents were tested but without satisfying results

CBZ with nearly neutral properties could generally be analysed without derivatization, but it was decomposed in the injector forming iminostilbene (IMINO) (Fig. 3.31). The derivatization of Glucu-SFM with diazomethane was proved by LC-ESI/MS. But due to elevated polarity the analysis with GC/MS was impossible.

The summarized results are presented as shown in (Table 3.3). Benzylation and methylation are chosen for further quantification method because they cover nearly the whole analytes.

In order to optimise the method, all parameters having direct influence in the yield of the reaction such as reaction time, reagent volume, reaction temperature and final solvent were investigated. The final modify methods are set as described in (section 5.2).

Based on the intention to get little by-products, derivatives of low molecular weight, short reaction times as well as lower detection limit, derivatization with diazomethane was chosen as the general method. The quantitative results from diazomethane are discussed in detail in section 3.3.2.

Table 3.3: Summarized results of the derivatization study

Analyte / Reagent	SFM	CBZ	IBU	DCF	Ac-SFM	Glucu-SFM	OH-IBU
Methylation	+	-	+	+	+	+	+
Benzylation	+	-	+	+	0	0	0
Silylation	-	-	+	+	0	0	0
Acylation	-	-	-	-	0	0	0

+ = suitable; high product yields
- = not-suitable; nearly no reaction products
0 = not tested

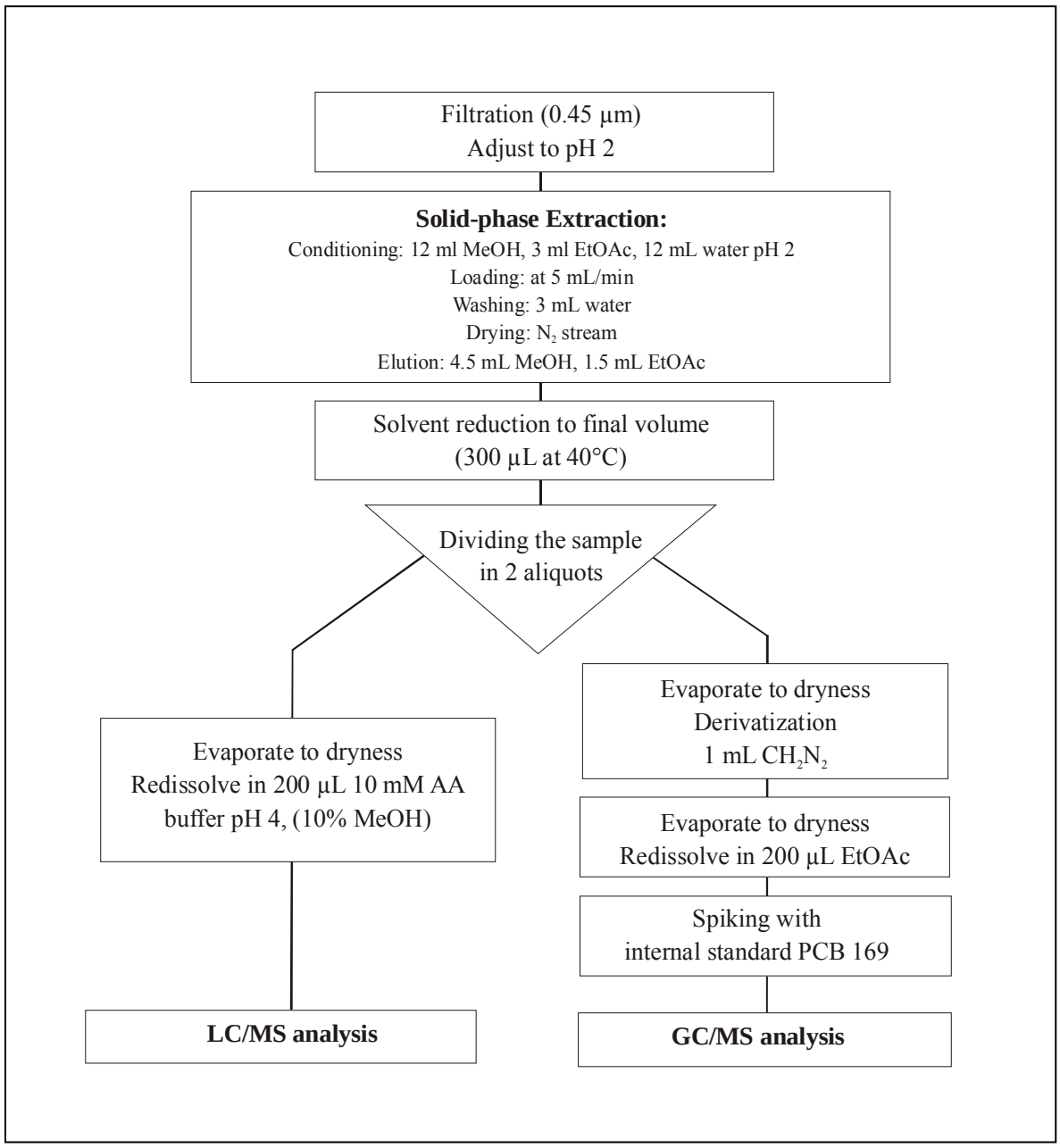
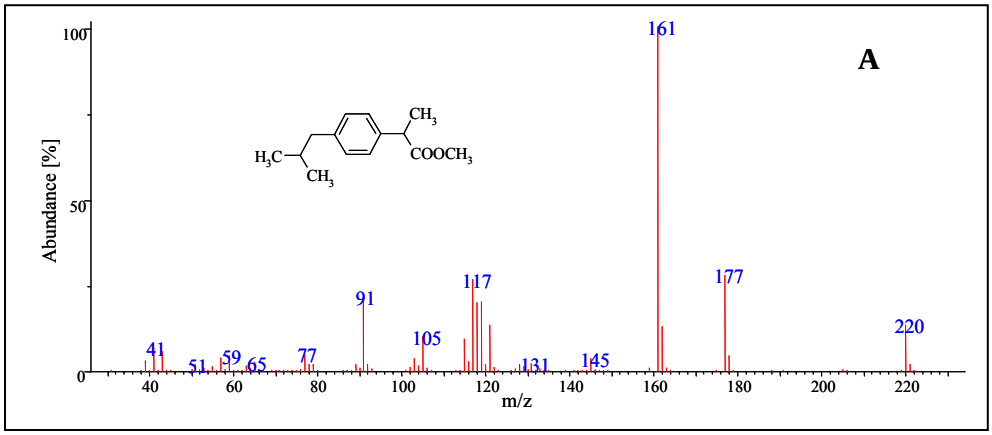


Fig. 3.27: Sample clean up and extraction scheme


A
Abundance [%]
m/z
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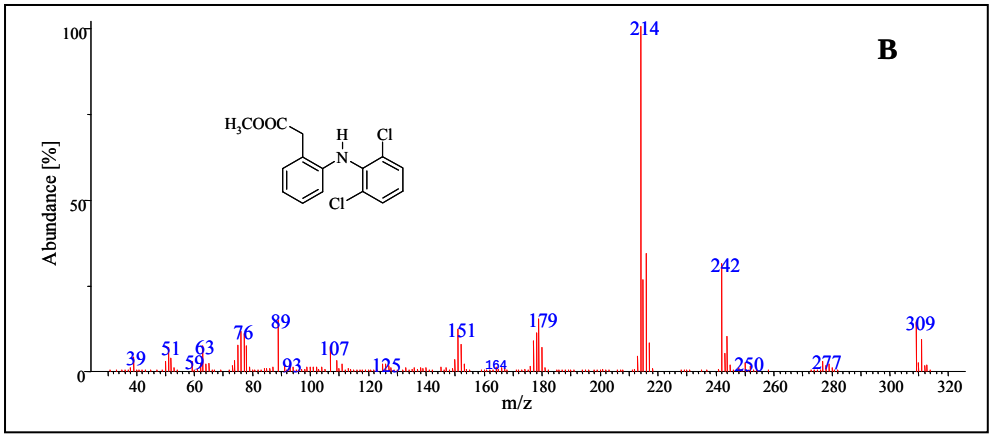
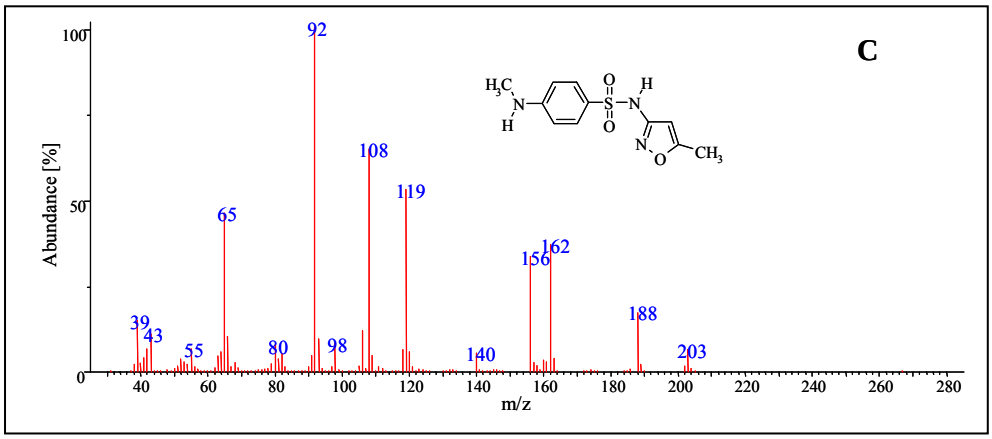
177
91
117
105
220
41
51
59
65
77
131
145
CH3
H3C
CH3
COOCH3


B
Abundance [%]
m/z
214
242
309
89
76
179
151
107
39
51
63
59
93
125
164
250
277
H3COOC
H
N
Cl
Cl


C
Abundance [%]
m/z
92
108
119
65
162
156
188
39
43
55
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98
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O
S
O
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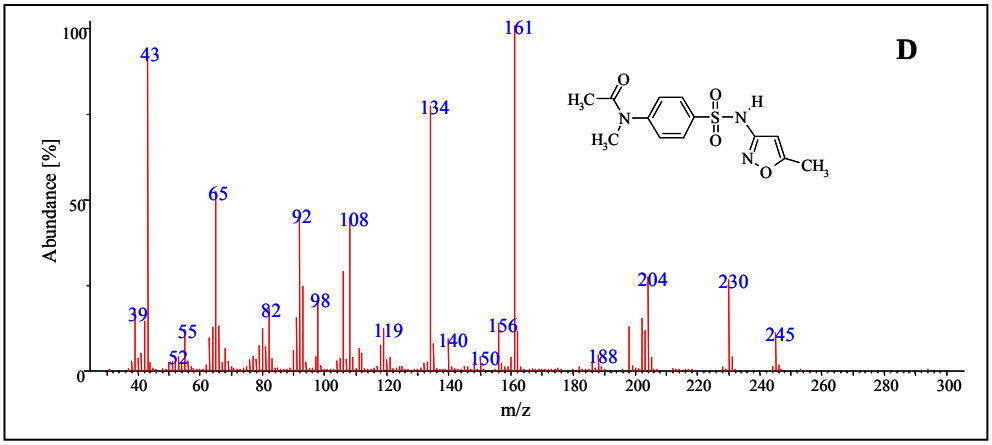
O
CH3



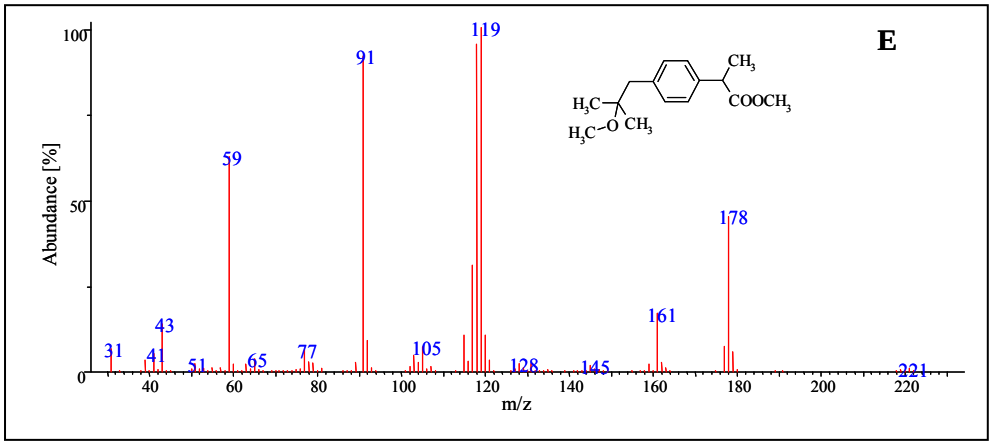
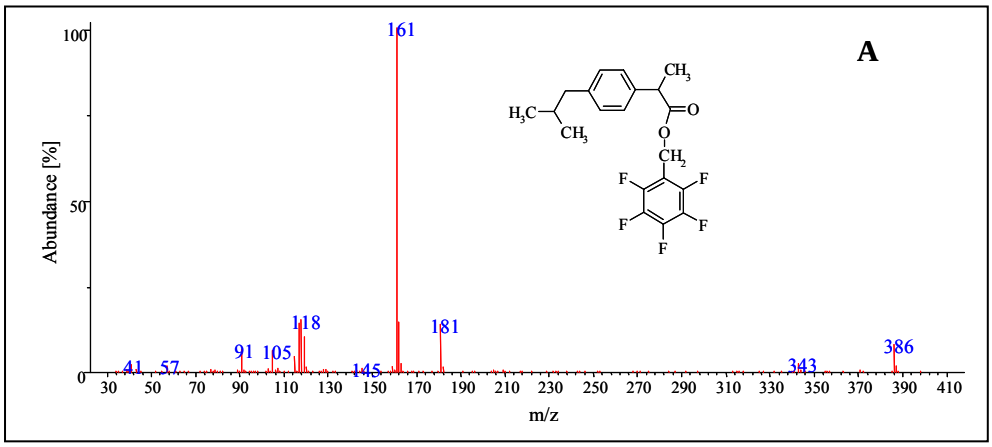
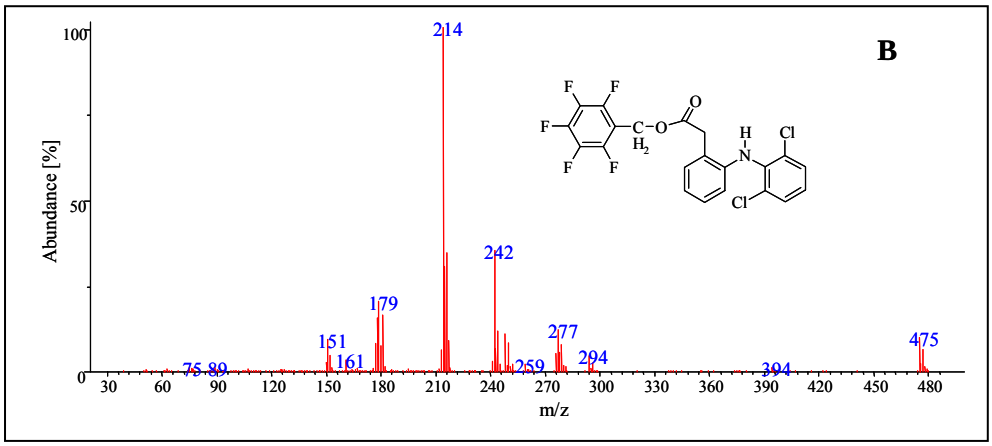


Fig. 3.28: Electron ionization mass spectra of the diazomethane derivative of the IBU (**A**), DCF (**B**), SFM (**C**), AC-SFM (**D**) and OH-IBU (**E**), the measurement was based on GC/MS using capillary column (HP-5MS), split/splitless injector and gradient temperature program as described in chapter 5





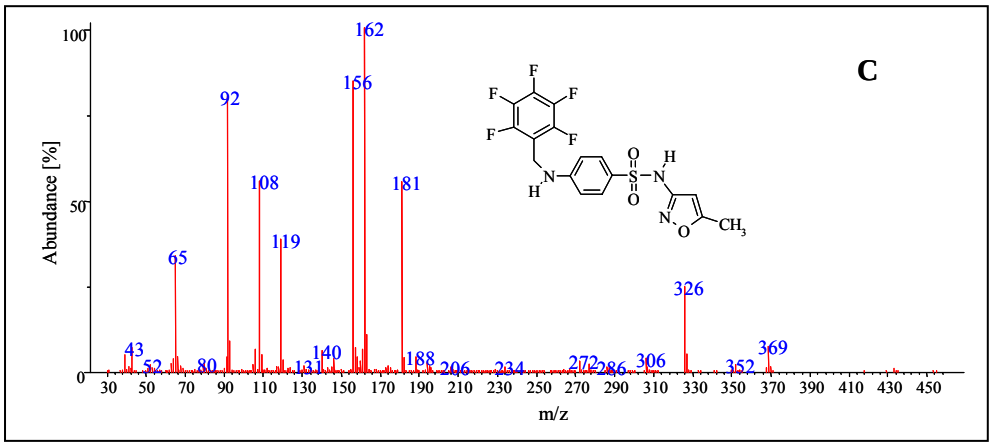
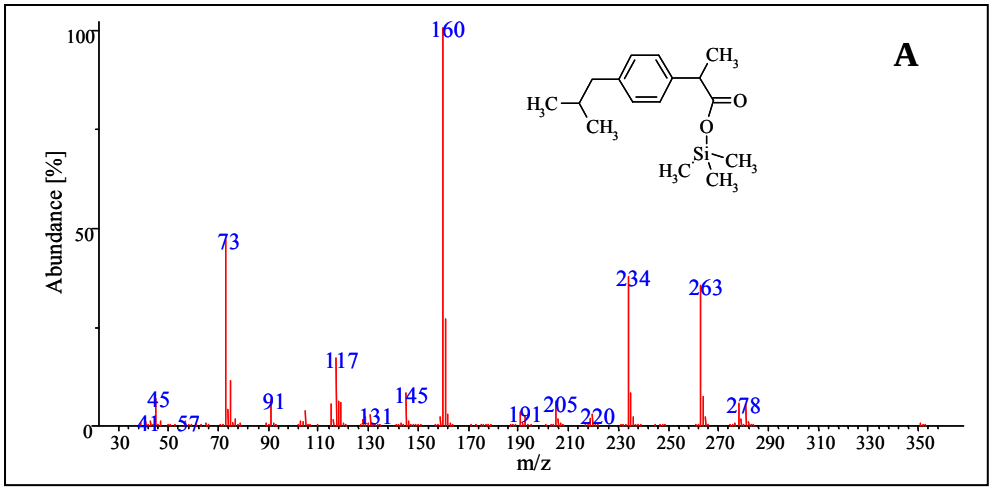


Fig. 3.29: Electron ionization mass spectra of the pentafluorobenzyl derivative of the IBU (**A**), DCF (**B**) and SFM (**C**)



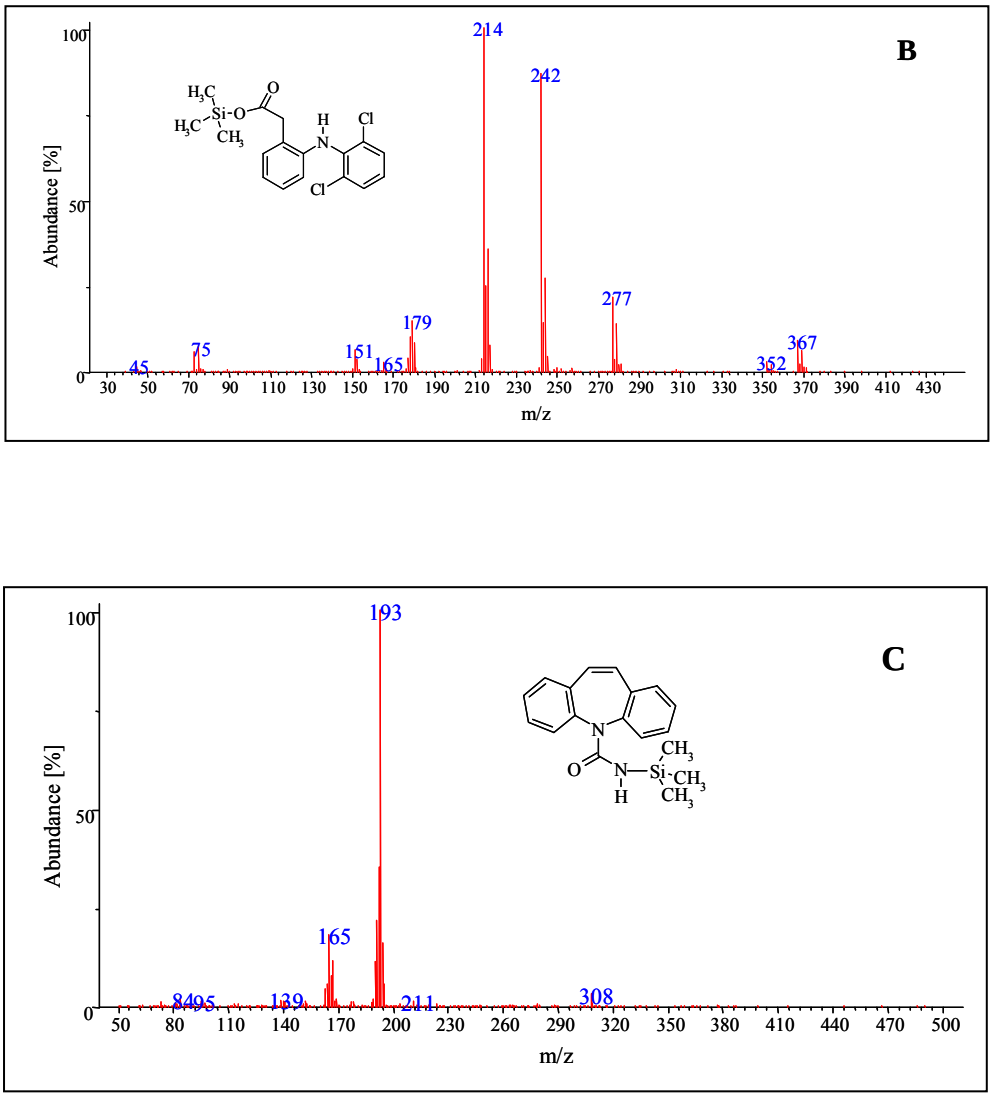
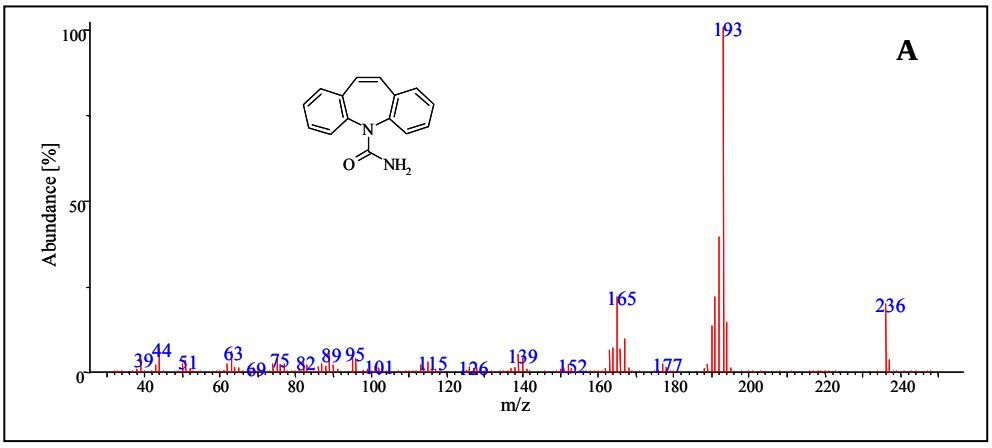


Fig. 3.30: Electron ionization mass spectra of the trimethylsilyl (TMS) derivative of the IBU (A), DCF (B) and CBZ(C)



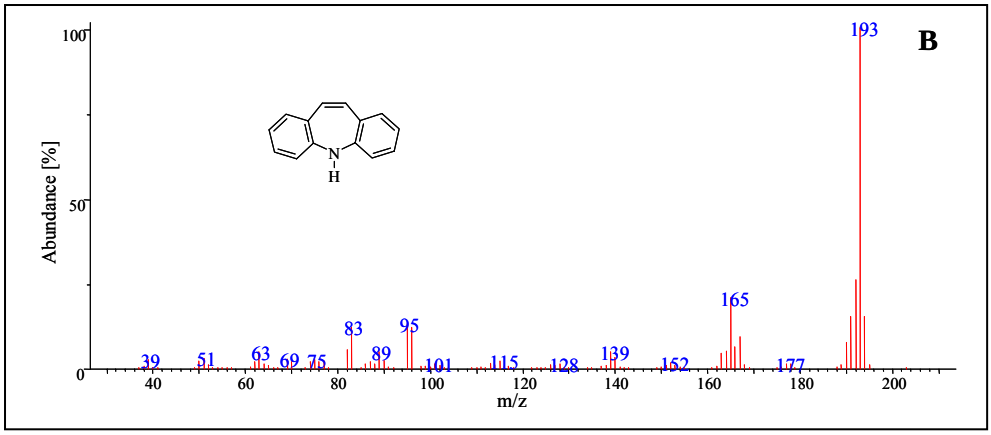


Fig. 3.31: Electron ionization mass spectra of CBZ (A) and IMINO (B)

3.3.2. GC/MS

Because of the high separation power of capillary columns followed by a specific detection by means of mass spectrometry the combined GC/MS technique is the most appropriate tool for volatile and thermal stable compounds.

All single parameters were varied and tested for the investigated pharmaceuticals including the main metabolites, which were synthesised at the University of Paderborn. The final working conditions based on methylation with diazomethane as described in chapter 5.

3.3.2.1. Determination of underivatized analytes

Direct analysis without derivatization was only possible for CBZ. Besides the signal for CBZ a second very sharp one with a shorter retention time could be observed, often more intensive than that of CBZ. It is caused by IMINO, a decomposition product of CBZ formed in the injector of the GC.

3.3.2.2. Methylation by diazomethane

As described in section 3.3.1 the analytical determination of the selected drugs was performed after derivatization with diazomethane as reagent for all analytes. The methylated products of IBU, OH-IBU, DCF, SFM, and Ac-SFM are shown in a total ion current (TIC) chromatogram (Fig. 3.32).

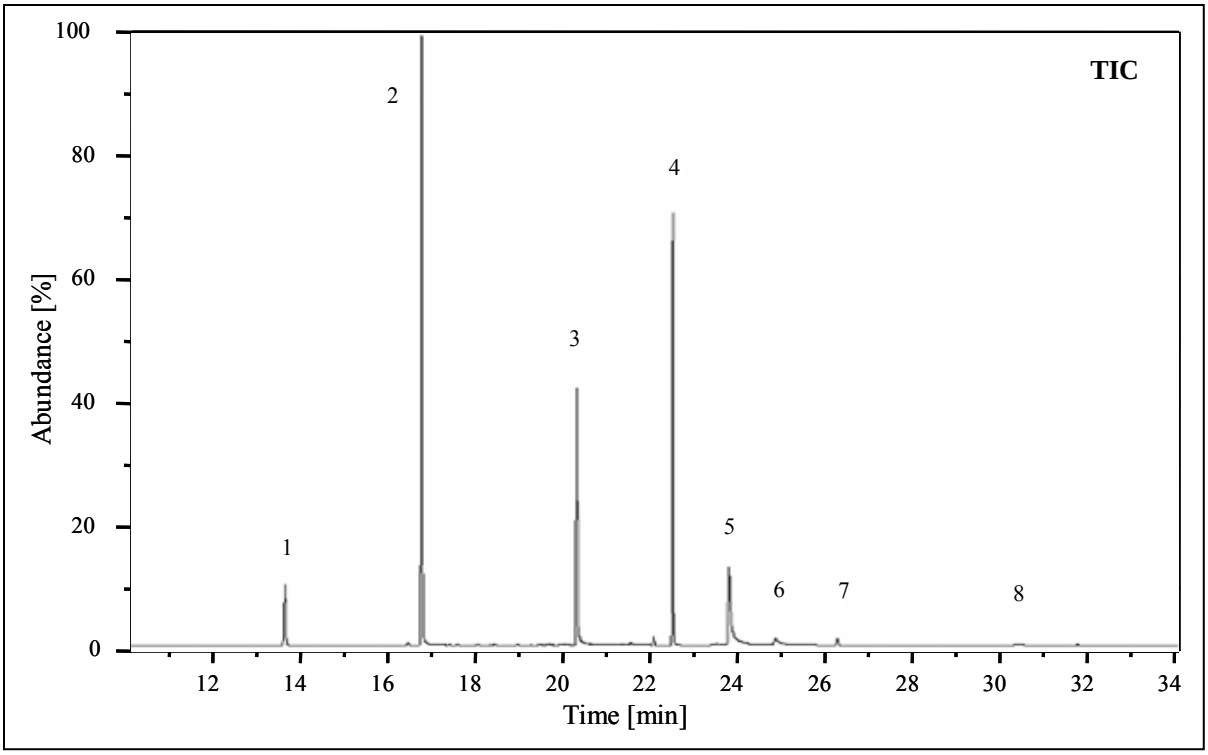


Fig. 3.32: GC/MS chromatogram of the methylated analytes (CH_2N_2), **1.** IBU-Me, **2.** OH-IBU-Me, **3.** IMINO, **4.** DCF-Me, **5.** CBZ, **6.** SFM-Me, **7.** PCB, **8.** Ac-SFM-Me, Using fused-silica capillary column (HP-5MS), split/splitless injector and gradient temperature program as shown in chapter 5

3.3.2.3. Fragmentation pattern of the methylated analytes

The chemical structure and typical mass spectra of the methylated derivatives are shown in Fig. 3.28 and the selected ions are listed in Table 3.4.

The mass spectrum of methylated **IBU** indicates a molecular ion $[M]^{+\bullet}$ at m/z 220, the major fragment ions appear at m/z 177 $[M\text{-}C_3H_7]^+$ and 161 $[M\text{-}COOCH_3]^+$. Further fragments appear at m/z 91, 105 and 119 indicating the structure of an alkyl-substituted aromatic system [35]. The mass spectrum of methylated **DCF** indicates a molecular ion $[M]^{+\bullet}$ at m/z 309, the major fragment ions are m/z 277 $[M\text{-}CH_3OH]^+$, 242 (further loss Cl), 214 (further loss CO) and 179 (further loss Cl) [59]. Though the mass spectrum of methylated **SFM** doesn't show a molecular ion $[M]^{+\bullet}$ at m/z 267, the methylated products could be validated by LC/MS as well as to Ac-SFM. The major fragments in GC/MS have m/z 203 (presumable loss of SO_2) and 188 (presumable further loss of CH_3). Further fragments are at m/z 156, 108 and 92. The mass spectrum of methylated **Ac-SFM** doesn't show a molecular ion $[M]^{+\bullet}$ at m/z 309, the major fragment ions appear at m/z 245 (presumable loss of SO_2), 230 (presumable further loss of CH_3), 161 (presumable loss of methyl-acetylaminophenyl) and 134 (acetylaminophenyl). Further fragments are at m/z 156, 108 and 92. The fragmentations pattern for both SFM and Ac-SFM are in agreement with data reported in the litreture [76].
The mass spectrum of methylated **OH-IBU** has only a weak molecular ion $[M]^{+\bullet}$ at m/z 236, the methylated products could be validated by LC/MS too. The major fragment ions are at m/z 178 $[M\text{-}58]^{+\bullet}$, 177 $[M\text{-}COOCH_3]^+$ and 118 (further loss of C_3H_7O). Further fragments have the m/z 161 and 91 [35, 77].

The underivatized mass spectra of **CBZ** as shown in Fig. 3.31a indicates a molecular ion $[M]^{+\bullet}$ at m/z 236, the major fragment at m/z 193 $[M\text{-}NHCO]^{+}$ and a further fragment at m/z 165.

3.3.2.4. Identification by SIM

Selected ion monitoring (SIM) is a technique in which a particular ion or a set of ions are selected and monitored. SIM experiments are useful for detecting small quantities of a target compound or to eliminate overlapping by signals of a complex mixture. The precondition is that the mass spectrum as well as the retention time of the target compound are known. Thus, SIM is useful for trace analysis for a rapid screening of a large number of samples for known target compounds.

Depending on the analyzer system of the mass spectrometer SIM can provide lower detection limits and greater speed of analysis than a full scan MS because only a few ions are monitored.

Therefore, quantification was performed by SIM using the most abundant and specific ions of each compound. The retention time - sometimes is an important help in identification - and the single ion masses of the selected pharmaceuticals are listed in (Table 3.4).

Table 3.4: Specific retention times of the analyte used for quantification in the (EI^{+}, SIM)

Analyte	R_t [min]	Molecular ion [m/z]	Ion 1 for SIM [m/z]	Ion 2 for SIM [m/z]
IBU-Me	13.63	220	161	117
OH-IBU-Me	16.72	236	178	119
IMINO	20.22	193	193	165
DCF-Me	22.39	309	214	242
CBZ	23.65	236	193	165
SFM-Me	24.71	267	156	92
PCB 169[a]	26.11	360	360	290
Ac-SFM-Me	30.10	309	161	134

a = instrument internal standard

3.3.2.5. LOD and LOQ

Instrument detection limit (IDL) is defined as the minimum concentration of the analyte that the instrument can detect with a signal to noise ratio 3:1. It is only a value for the efficiency of the instrument and, consequently, independent from sample preparation. The method detection limit (MDL) is similar to an IDL, but is based on samples, which passed all single method steps. A limit of quantitation (LOQ) is normally 6 to 10 times the MDL value and is considered to be the lowest concentration that can be accurately measured. DLs are actually determined by analysis of multiple low-level samples in addition to blanks. This information gives the variation in instrument response at levels near the detection limit.
The IDL and instrument LOQ were determined by measuring standard solutions of the analytes in the concentration range of 1-10 µg/L and in addition blank samples as well. Whereas, MDL and method LOQ were determined by spiking the analytes into 1 L ultrapure

water sample in the concentration range 1-10 ng/L, leading to an enrichment factors of 2500 after sample preparation steps. In case of SFM and Ac-SFM this range could not be achieved.

The MDL and LOQ investigation gave different results based on the chemical structure characteristics of the analytes. Acidic and neutral compounds showed MDLs between 1-5 ng/L and LOQs between 3-10 ng/L (Table 3.5), but the polar compounds, e.g. SFM, have relatively higher detection limits up to 100 ng/L. The reason might be interpreted due to the efficiency of the enrichment and derivatization procedures. Therefore, gas chromatography is not the most suitable method for SFM and its metabolites especially in a lower concentration range. For neutral and acidic analytes, IDL and MDL are 1-5 and 2-12 pg absolute per injection respectively.

Table 3.5: Regression coefficients, limits of detection and limits of quantification obtained in SIM mode of GC/MS, * = underivatised

Analyte	r^2 DW[a]	r^2 SW[b]	IDL [pg]	I-LOQ [pg]	MDL [ng/L]	M-LOQ [ng/L]
IBU-Me	0.996	0.9982	1	2.5	5	10
OH-IBU-Me	0.9985	0.9998	1	2	1	3
DCF-Me	0.9989	0.9998	1	2.5	3	5
CBZ*	0.9982	0.9987	5	10	5	10
SFM-Me	-	-	50	100	100	250
Ac-SFM-Me	-	-	500	750	500	1000

a = 1 L sample (distilled water), b = 0.1 L sample (surface water), I = instrument, M = method

3.3.2.6. Calibration

Stock solutions were prepared by solubility the analytes in ultrapure water. Different concentrations were prepared by dilution the concentrations solution in ultrapure or real waters. All stock solutions were stored in the refrigerator to protect them against photodegradation. They were warmed up to room temperature before use.

Quantification was performed using external calibration. Calibration curves were created from 6 concentration points (n=3) in the concentration range 10-1000 ng/L. The sample volume was 1.0 L using ultrapure water and 0.1 L using surface water (river Ruhr). GC/MS data acquiring was done by the SIM technique. The peak areas were corrected due to a background concentration of the analytes in the real water samples. The peak areas were plotted against the corresponding concentration of the analytes (Fig. 3.33).

Sample volumes of 100-1000 mL were extracted, leading to enrichment factors ranging between 250 and 2500 in order to test the influence of the volume. As mentioned before in the derivatization section CBZ partially formed IMINO as a thermal degradation product caused by the GC injector. Therefore, the sum of both areas was accumulated as one unit for calibration.

Based on MDL and LOQ, the methylated form of SFM, Ac-SFM, and Glucu-SFM were excluded from GC/MS calibration. An alternative procedure using LC-ESI/MS/MS is described later in section 3.3.3.

The quality of the developed method was confirmed by the linearity of the calibration curves up to 1 µg/L (Fig. 3.33). No further investigation was done for higher concentrations because the analytes expected to be present in a relative low concentration range in surface water. In a case of higher concentration, such as in wastewaters, the samples could be fitted by dilution. Regression coefficients are > 0.99 in most cases, indicating a good linearity of the calibration curves (Table 3.5).

In order to have an overview about the GC/MS instrument reproducibility, 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) was spiked as an internal instrument standard after the derivatization step. From numerous injections within a period of weeks, the relative standard deviation was found to be lower than 4 %. In GC/MS all analytes except IBU showed high precise measurements. The reason may be the sample handling that means a loss of an amount during the drying step in the heating block under a stream of nitrogen.

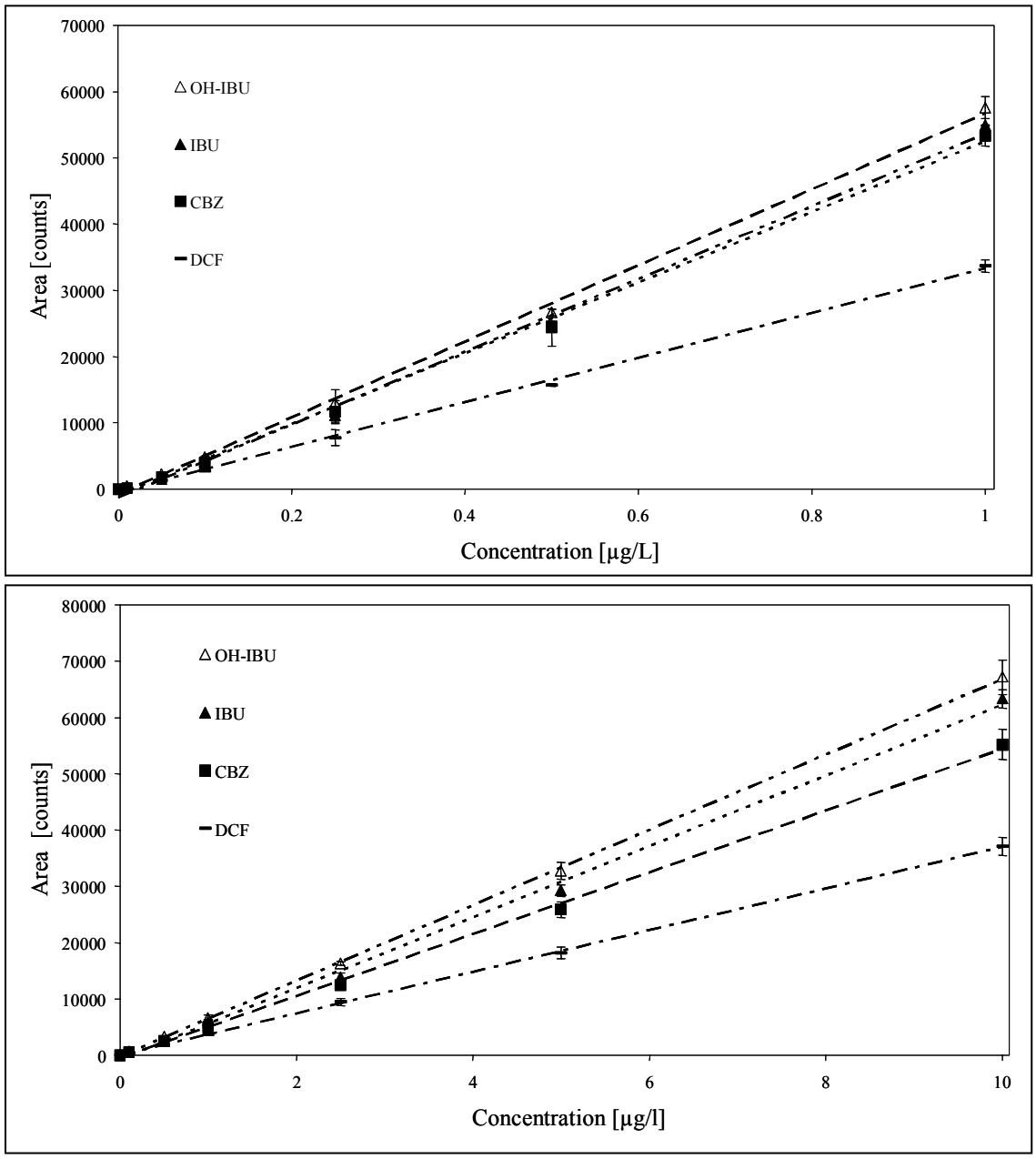


Fig. 3.33: Calibration curves from GC/MS analysis after SPE and derivatization with CH_2N_2 (A = 1 L spiked ultrapure water, B = 0.1 L spiked river water; n=3)

3.3.3. LC/MS

Though the liquid chromatography has a restricted separation power in comparison to capillary GC and though the combination with mass spectrometry is not the best support for compound identification because of fragment-poor spectra, this technique is indispensable in the analysis of polar and thermo-labile analytes. The possibility to apply the MS/MS-technique to verify aimed fragments increases the relevance of this method immensely.

3.3.3.1. Separation characteristics

To achieve reliable analytical separations in LC different mobile phases and the influence of the pH were tested. The pH-value can have a great influence in the retention time as it is demonstrated for DCF and IBU in (Fig. 3.34), while SFM and CBZ have nearly constant retention time in the whole pH-range. To achieve an optimum in separation power and to reach high sensitivity a method based on capillary LC-ESI/MS was developed. The experimental conditions are collocated in chapter 5.

In all cases a compromise has to be made between the improvement of separation and deterioration of the ionization process. Therefore, many mobile phase compositions with different pH values were studied to achieve suitable chromatographic separation and a stable ionization spray. Methanol is a better protic solvent and generally produces more ions than acetonitrile. Whereas, acetonitrile lead to better HPLC separation and a lower column back pressure. Initially, both acetonitrile and methanol were tested as organic mobile phases for LC separation. The measurements were finally carried out with a mixture of both of them. An adequate mobile phase, as discussed later in section 5.3, led to superior composition for interface ionization, shorter retention time and better resolution of the analytes.

Fig. 3.35 shows the extracted mass chromatogram of the target pharmaceuticals and their metabolites after the SPE enrichment and HPLC separation. Under the described conditions all analytes were resolved chromatographically within a retention time of 23 min.

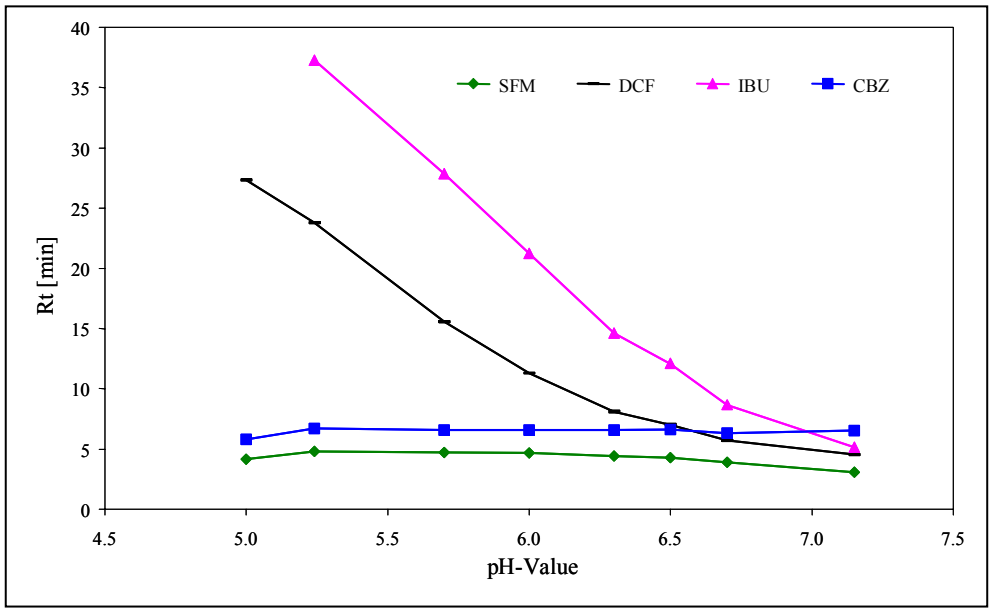


Fig. 3.34: Influence of the HPLC mobile phase pH on retention times

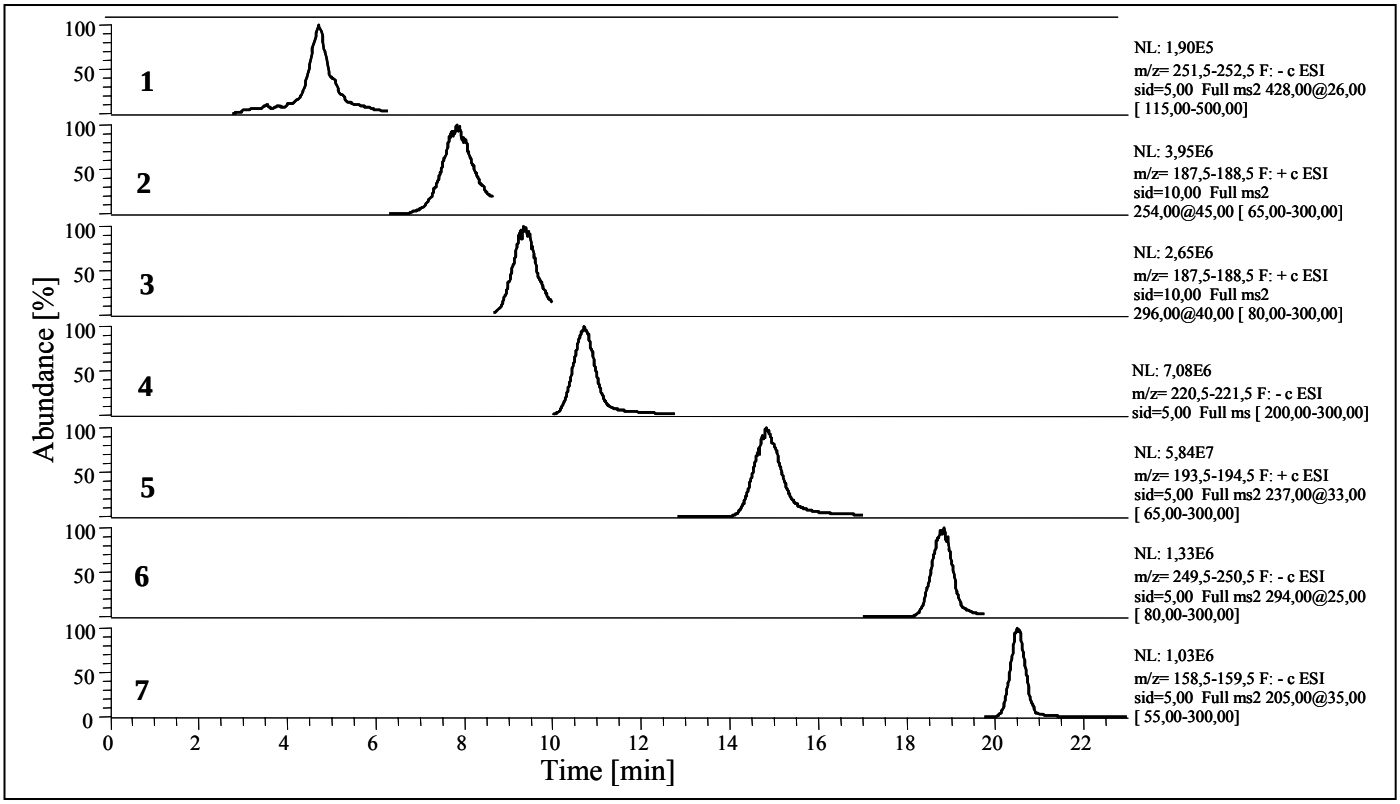


Fig. 3.35: Extracted mass chromatograms from conventional LC-ESI/MS analysis of standard solution **1.** Glucu-SFM (ESI^-), **2.** SFM (ESI^+), **3.** Ac-SFM (ESI^+), **4.** OH-IBU (ESI^-), **5.** CBZ (ESI^+), **6.** DCF (ESI^-), **7.** IBU (ESI^-), using Aquasil C-18 as analytical column and a gradient program as shown in chapter 5 from mobile phase A and B

3.3.3.2. Mass spectrometer parameters

All analyses were carried out with an electrospray ionisation (ESI) interface and an ion trap mass analyzer. Instrument control, data acquisition and evaluation were done with Xcalibur software.
Several mass spectrometric parameters had to be optimised in order to obtain the highest possible abundance of the analytes in MS. With ESI most of the ions are already formed in the liquid phase and thus the eluent composition has a significant role in the ionisation process. Optimisation of the eluent composition is discussed in the previous section. The first parameters to be optimised in ESI are those having a crucial effect on the spray formation, namely the voltage of the capillary and the flow rate of the nebulising gas.

Electrospray operation parameters were optimized by direct infusion of the analytes by means of a syringe pump at a flow rate of 10 µL/min into the ESI source. Nitrogen was used as sheath gas at a flow rate of approximately 0.3 L/min, the spray voltage was set to 3.5 kV in the positive ionization mode and to 3.2 kV in the negative ionization mode, the transfer capillary was set to 200 ˚C.

Successive work was done to optimize the spray voltage and transfer capillary; generally, low voltage leads to a smaller detector response, because the formed droplets carry less charge, a too high value causes arcs in the source. To obtain the best sensitivity for the detection, different capillary temperatures were investigated in order to assist the dissolution of the ions produced by ESI. 200 ˚C was the optimum value because an increasing temperature showed

degradation of DCF. Isopropanol/water (5:1) was used as sheath liquid at a flow rate of 4 µL/min in order to stabilize and enhance the ESI signal.
Following the optimization of capillary voltage, ion optics parameters were optimized for each polarity mode based on mass peak intensity. After the method development was finished, the formation of ammonium adducts in the positive mode and acetate adducts in the negative mode were observed. Moreover, oligomers were formed at a higher concentration in the both ionization mode. It is often observed that adduct ions hamper qualitative and quantitative results, especially when unexpected. These are formed by either association between species present in the mobile phase system that are preserved due to the soft ionization of the electrospray process or to the presence of gas-phase collisions in the spray chamber prior to sampling by the mass spectrometer. Thereby, the most important parameter is the declustering potential, which should be tuned in order to reduce the formation of adducts and to increase the abundance of the parent ions. To dissociate as much as possible from those adducts without any dissociation of the parent ions, an ion source collision energy was applied in the skimmer region. The optimal collision energy for those compounds was between 5 and 10 V in negative and positive ion mode (Table 3.6). The analytes were detected using a time-segments mode with positive and negative voltage switching.

The properties of the functional groups define the ionisation mode; DCF, IBU and OH-IBU contain carboxylic acid, so they will favor the negative mode while CBZ, SFM, Ac-SFM and Glucu-SFM prefer the positive mode because of their amine functionality. Due to their structural ambivalence of SFM and its metabolites, the detection was possible in negative ionization mode as well and for Glucu-SFM it was also favorably.

Considering Glucu-SFM, ESI is specially favourable because it operate without heat input in the spray-ionization step, thus allowing the polar glucuronide to be ionized without thermal degradation [78].

3.3.3.3. Confirmation by MS/MS

For the analysis of complex mixtures, the MS/MS feature - also called ‘selected reaction monitoring’ (SRM) - provides a high degree of selectivity, specificity, and sometimes a better limit of detection than full scan MS. In SRM scan mode one species of ions, called parent ions, are selected and stored in the mass analyzer. Then these ions are excited by energy so that they collide with the background gas atoms. By that, the parent ions fragment to one or more product ions, which are recorded in an SRM product ion mass spectrum. This procedure can be repeated several fold. Like SIM, SRM is of advantage for a rapid analysis of trace components in complex mixtures.

At first, the optimum parameters of the MS/MS acquisition had to be determined for each individual compound, in order to characterize the typical fragmentation pattern and to maximise abundances of fragment ions for each compound. It was performed using an online injection valve; 20 µL (1 mg/L) standard solution containing the analytes was injected into the mobile phase effluent of the HPLC; 50 % of buffer B at a flow rate of 13µL/min. After the selection of the parent ions by mass analyser, the collisions of the parent ions caused the fragmentation producing the product ions. After determination of the most significant product ions, optimisation of the collision energy was carried out to produce the maximum abundance of product ions. Parent and product ion masses of the individual compounds are given in (Table 3.6).

Table 3.6: Optimized LC-ESI-MS/MS parameters for the analysis of the pharmaceuticals and some their metabolites

Analyte	R_t [Min]	Mode	Collision energy [V]	SRM [m/z]	Normalized collision energy [%]	Fragment ion [m/z]
Glucu-SFM	4.6	-ve	5	428	26	252/175
SFM	7.6	+ve	10	254	45	188/156
Ac-SFM	9.2	+ve	10	296	40	236/188
OH-IBU	10.7	-ve	5	221	0	-
CBZ	14.8	+ve	5	237	33	194
DCF	19.1	-ve	5	294	25	250
IBU	20.7	-ve	5	205	35	159

3.3.3.4. Analyte fragmentation pattern

All analytes were measured in full scan mode and under MS/MS conditions.

The mass spectrum of **CBZ** contains an abundant $[M+H]^+$ ion at m/z 237 and only a small fragment ion at m/z 194 (Fig. 3.36A). Fig 3.36B shows the MS/MS product ion spectra of the $[M+H]^+$ ion; as expected the collision results in the structural fragment at m/z 194 which corresponds to the loss of HNCO. The mass spectrum of **SFM** contains an abundant $[M+H]^+$ ion at m/z 254 and a weak ammonium adduct $[M+NH_4]^+$ (Fig. 3.37A). Fig 3.37B shows the MS/MS product ion spectra of the $[M+H]^+$ ion with m/z 188 which corresponds to rearrangement by losing H_2SO_2 [79]. Other minor fragments appear at m/z 156, 108 and 92; the interpretation for these fragmentations is shown in (Fig. 3.38) and affirmed by literature [80]. The mass spectrum of **Ac-SFM** contains an abundant $[M+H]^+$ ion at m/z 296 and a weak ammonium adduct $[M+NH_4]^+$ (Fig. 5.39A). Fig 5.39B shows the MS/MS product ion spectra of the $[M+H]^+$ ion with m/z 188 which corresponds to the loss ($CH_3CO+HSO_2$). Other fragments appear at m/z 230 (loss of H_2SO_2), 198 and 136; the interpretation of these fragmentations follows the fragmentation of the SFM pattern as shown in (Fig 3.38) [23,80]. The mass spectrum of **Glucu-SFM** contains an abundant $[M-H]^-$ ion at m/z 428, the other ion at m/z 252 corresponds to deprotonated SFM formed by the cleavage of the glycoside bond (Fig. 3.40A). Fig. 3.40B shows the MS/MS product ion spectra of the $[M-H]^-$ ion with m/z 252 which corresponds to the loss of the glucuronide moiety. The mass spectrum of **DCF** contains an abundant $[M-H]^-$ ion at m/z 294 and only a small fragment ion with m/z 250 (Fig. 3.41A). Fig 3.41B shows the MS/MS product ion spectra of the $[M-H]^-$ ion of DCF with m/z 250, corresponding to the expulsion of CO_2 [81]. The mass spectrum of **IBU** contains an abundant $[M-H]^-$ ion at m/z 205 in addition to the acetate adduct $[M-H+CH_3COOH]^-$ and the dimer $[2M-H]^-$ (Fig. 3.42A). Fig. 3.42B shows the MS/MS product ion spectra of the $[M-H]^-$ ion of IBU with m/z 159 which corresponds to the loss of ($CO+H_2O$). The mass spectrum of **OH-IBU** contains an abundant $[M-H]^-$ ion at m/z 221 in addition to the acetate adduct $[M-H+CH_3COOH]^-$ and the dimer $[2M-H]^-$ (Fig 3.43). The MS/MS experiment for OH-IBU showed unstable product ions at m/z 177, 159 and 133.

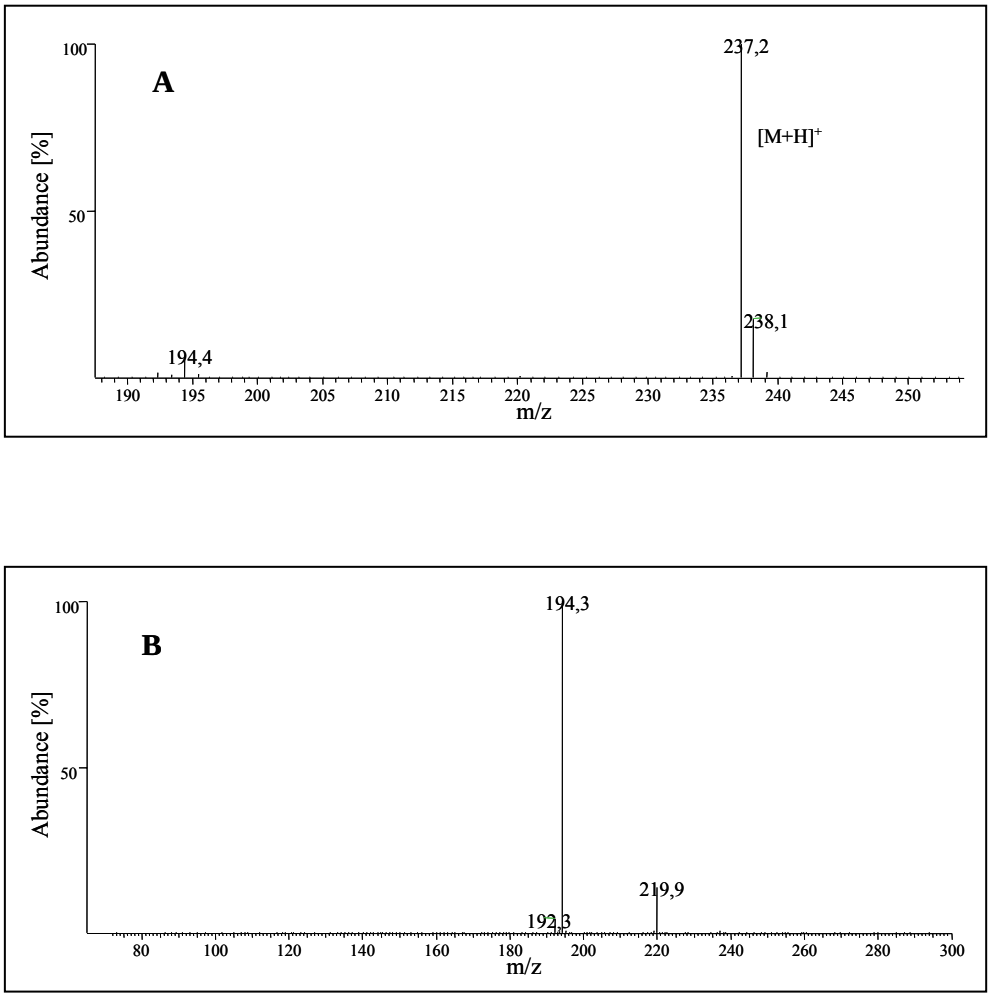
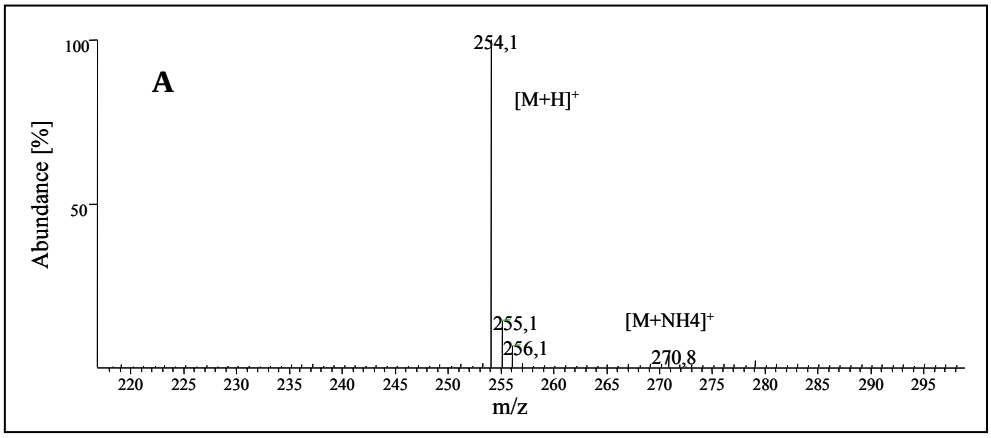


Fig. 3.36: LC-ESI/MS spectrum of CBZ (A) and the MS/MS product ion spectrum of the $[M+H]^+$ ion at m/z 237 (B)



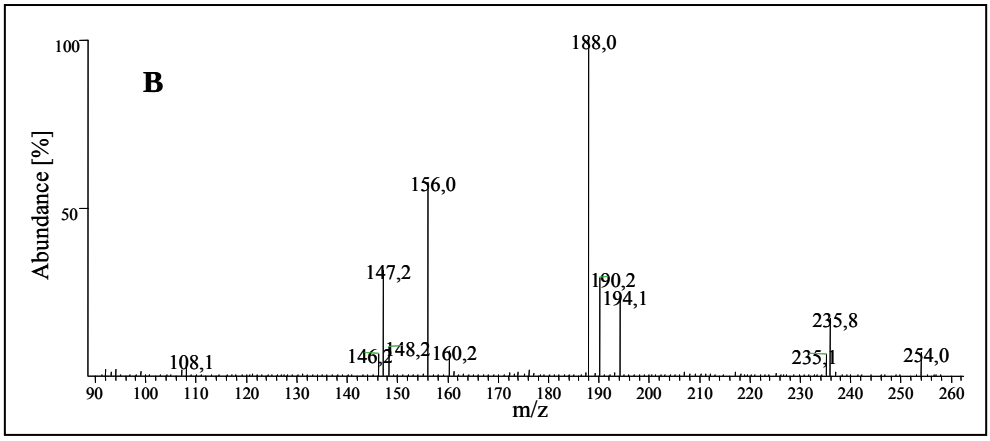


Fig. 3.37: LC-ESI/MS spectrum of SFM (A) and the MS/MS product ion spectrum of the $[M+H]^+$ ion at m/z 254 (B)

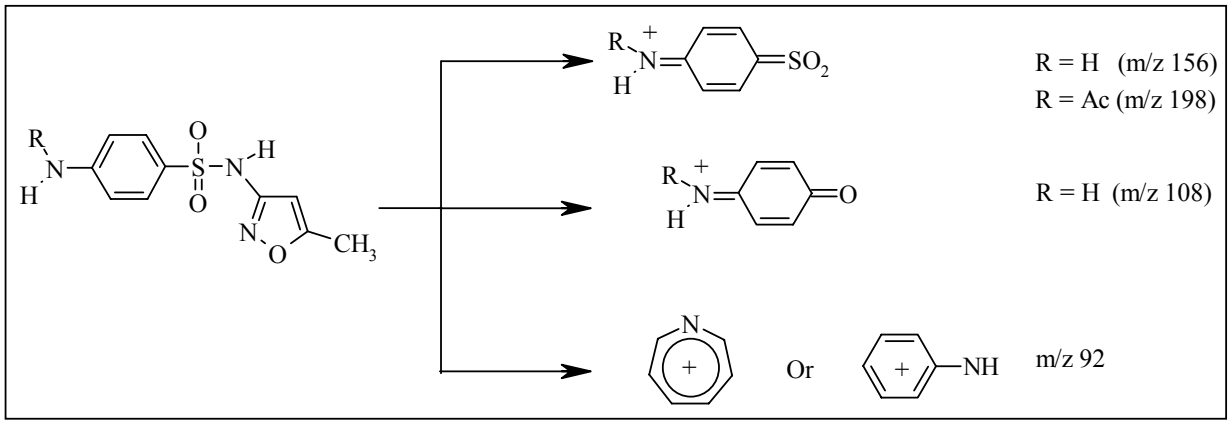
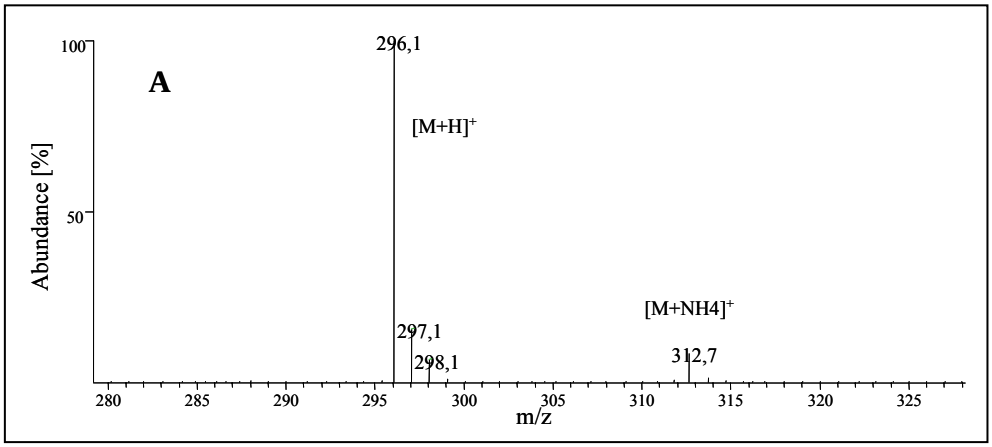


Fig. 3.38: Common CID fragmentation reaction for SFM and Ac-SFM



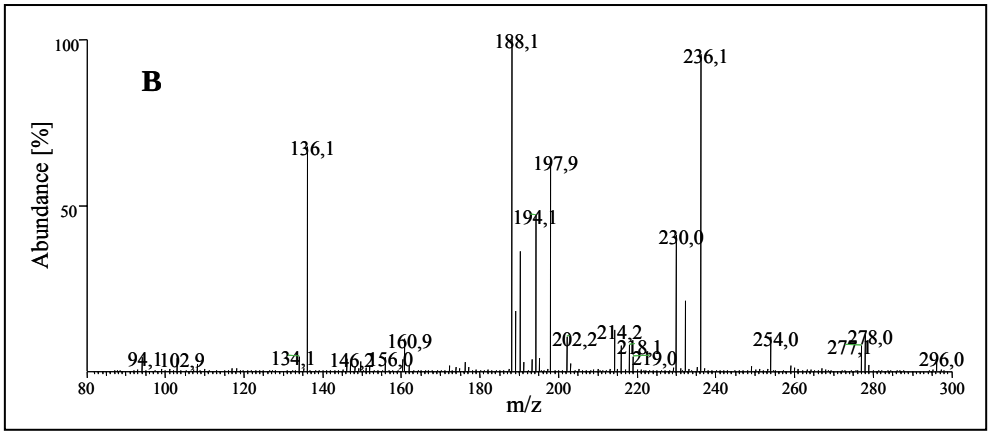


Fig. 3.39: LC-ESI/MS spectrum of Ac-SFM (A) and the MS/MS product ion spectrum of the $[M+H]^+$ ion at m/z 296 (B)

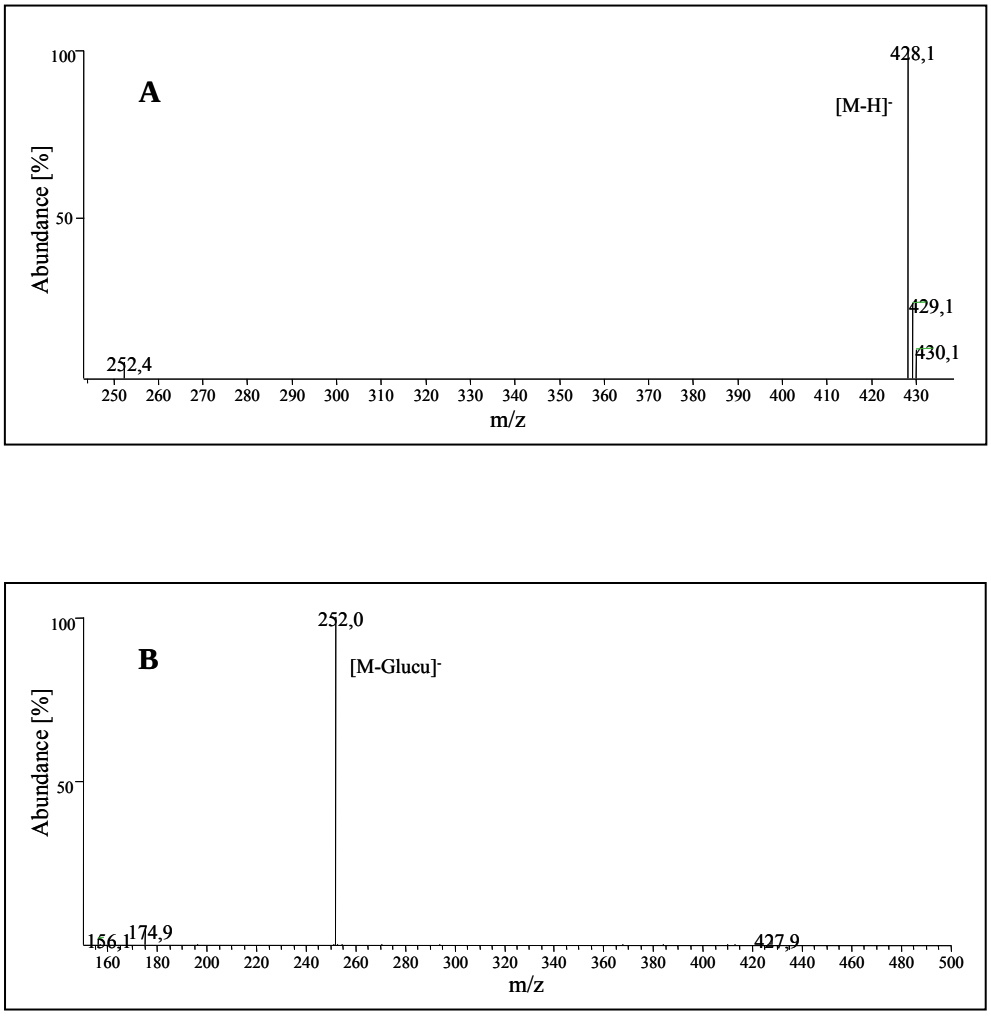


Fig. 3.40: LC-ESI/MS spectrum of Glucu-SFM (A) and the MS/MS product ion spectrum of the $[M-H]^-$ ion at m/z 428 (B)

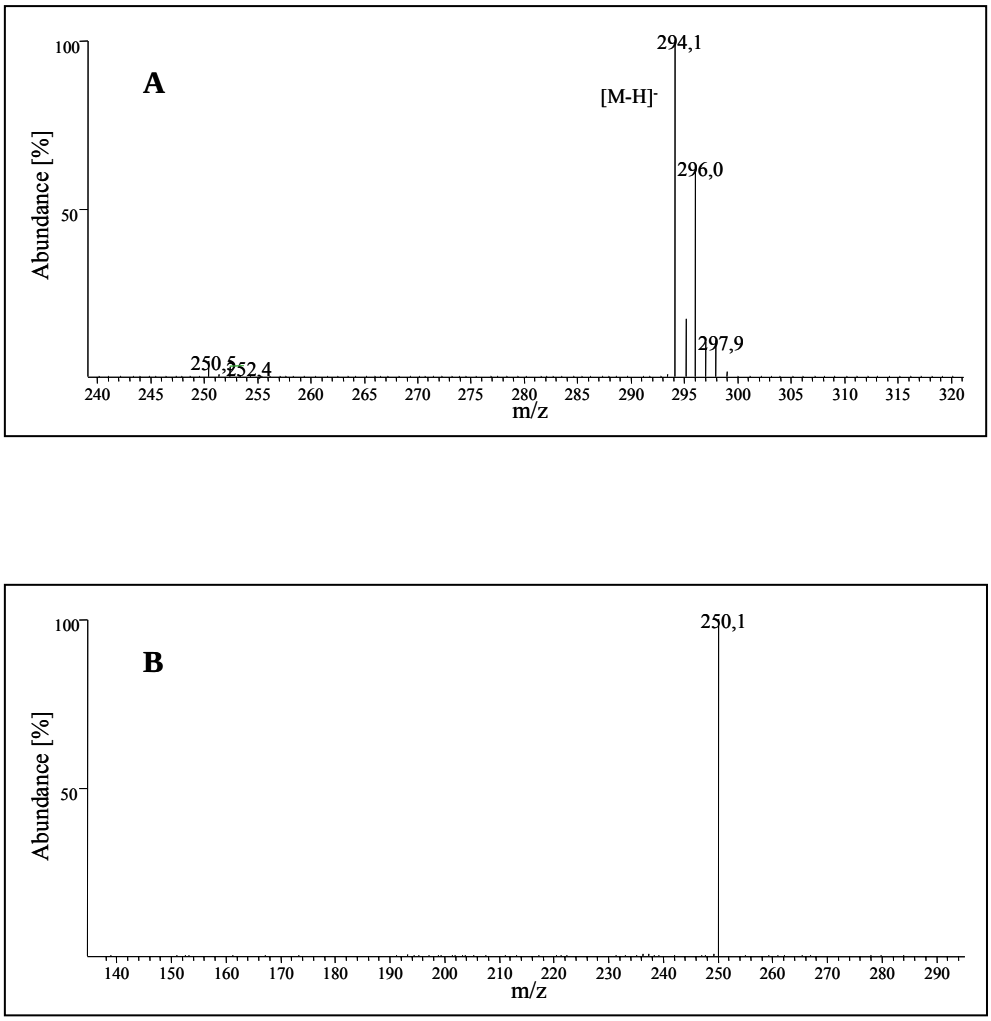
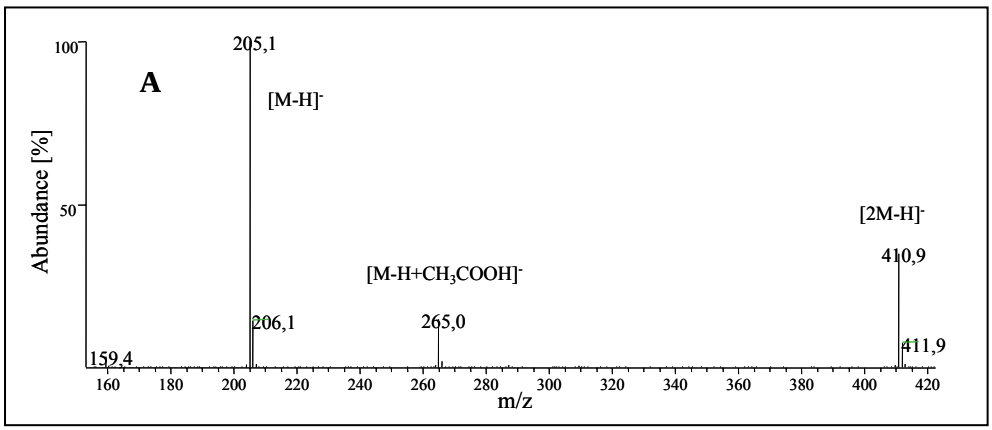


Fig. 3.41: LC-ESI/MS spectrum of DCF (A) and the MS/MS product ion spectrum of the $[M-H]^-$ ion at m/z 294 (B)



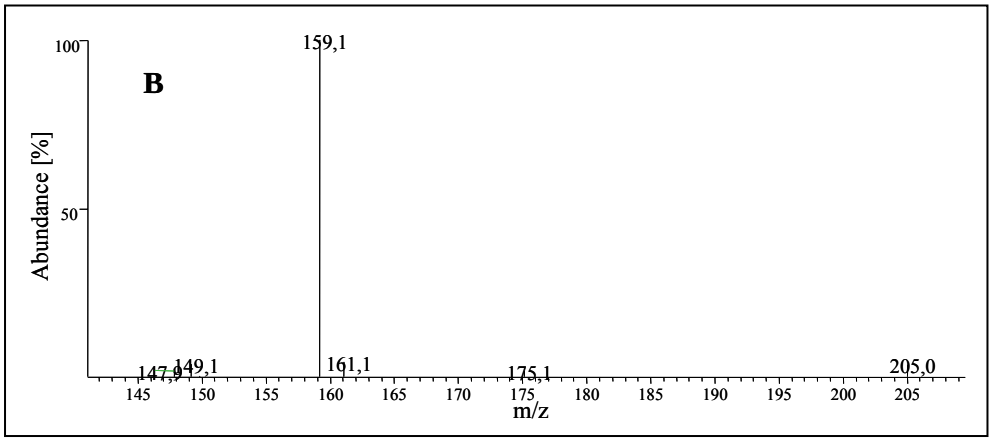


Fig. 3.42: LC-ESI/MS spectrum of IBU (A) and the MS/MS product ion spectrum of the $[M-H]^-$ ion at m/z 205 (B)

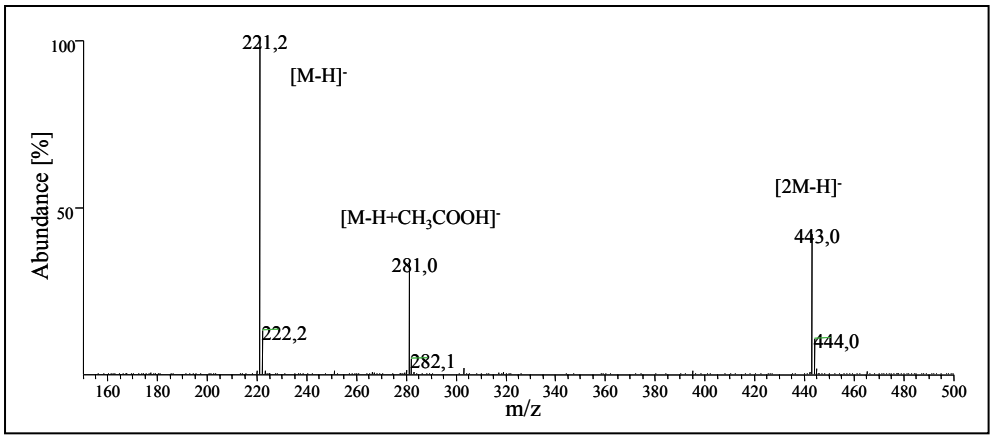


Fig. 3.43: LC-ESI/MS spectrum of OH-IBU

3.3.3.5. LOD and LOQ

The particular definitions are given in the GC/MS chapter (3.3.2.5). The quantification limits are a function of chromatographic conditions, mass spectrometry parameters and chemical characteristics of the analyte, and in LC especially the eluent composition because of the mobile phase modifiers. Moreover, analyte features (i.e. hydrophobicity, lacking polarity) can improve the ionization efficiency; thereby, instrument DLs can decrease significantly. MLD and quantification follow the same criteria.

Instrument DLs varies between 20 and 140 pg absolute for all analytes. LOQs are distributed between 60 and 200 pg absolute (Table 3.7). The analyte showed MDLs between 3-5 ng/L and LOQ between 5 –10 ng/L.

Table 3.7: Regression coefficients, rounded limits of detection and limits of quantification obtained in LC-ESI/MS method

Analyte	r^2 DW[a]	r^2 SW[b]	IDL [pg]	I-LOQ [pg]	MDL [ng/L]	M-LOQ [ng/L]
Glucu-SFM	0.9994	0.9774	100	200	5	10
SFM	0.9987	0.9996	20	60	5	10
Ac-SFM	0.9988	0.998	80	120	5	10
OH-IBU	0.9986	0.9996	60	120	5	10
CBZ	0.9878	0.998	60	120	3	5
DCF	0.9987	0.9998	140	200	5	10
IBU	0.9991	0.997	100	200	5	10

a = 1 L sample (distilled water), b = 0.1 L sample (surface water), I = instrument, M = method

3.3.3.6. Calibration

LC-ESI/MS quantification was performed using external calibration. The calibration curves were built from 6 concentrations (n=3) in a measurement range of 10-1000 ng/L by spiking 1.0 L ultrapure water and 0.1 L river water samples with the analytes; the enrichment factors were ranging between 250 and 2500 after sample preparation steps.

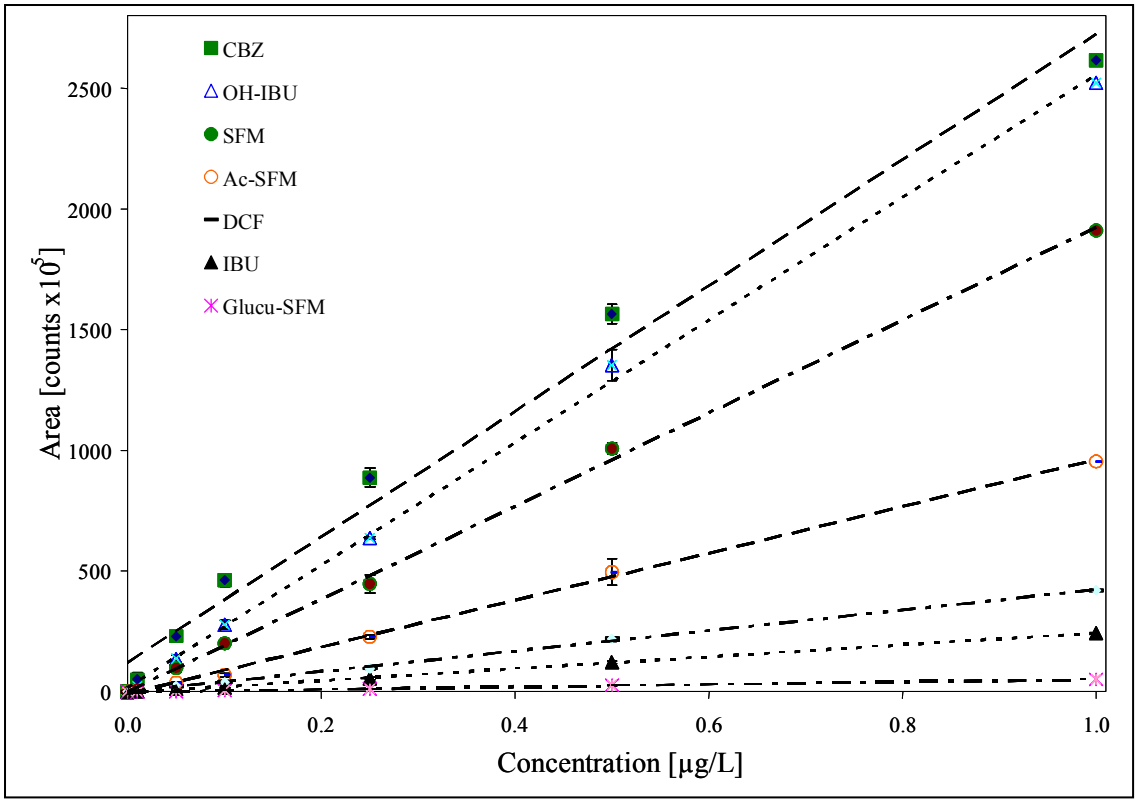
When the analyte concentration is too high for the capillary column a shift in the retention time can be observed. In order to solve this problem the injection volume was decreased from 20 µl to 10 µl and additionally the column equilibration time was extended.

The acquisition by LC-ESI/MS method was based on MS/MS experiments except for OH-IBU. In this case the full scan mode was used because the fragmentation product ions are instable. For the real water samples the analyte peak areas were background corrected. The high linearity of this method could be demonstrated for the selected calibration range (Fig. 3.44). No further investigation was done with higher concentrations because the selected analytes are present in a relative low concentration range in surface water. In the case of higher concentration, the sample could be diluted. The regression coefficient was > 0.97 in most cases, indicating a good linearity of the calibration curves (Table 3.7).

LC-ESI/MS instrument reproducibility was studied by successive injections of the same sample, the RSD was found to fluctuate a little bit from day to day, which is quite normal in LC-ESI/MS and probably a result of the instability of the spray. To solve this problem, an internal standard (e.g. isotope label compounds) should be used in order to compensate the uncontrolled random errors caused by other components in the system or the instrument itself.

Isotopes labeled compounds of our selected drugs are not available. Therefore, no internal standards were included in this investigation.

A)



B)

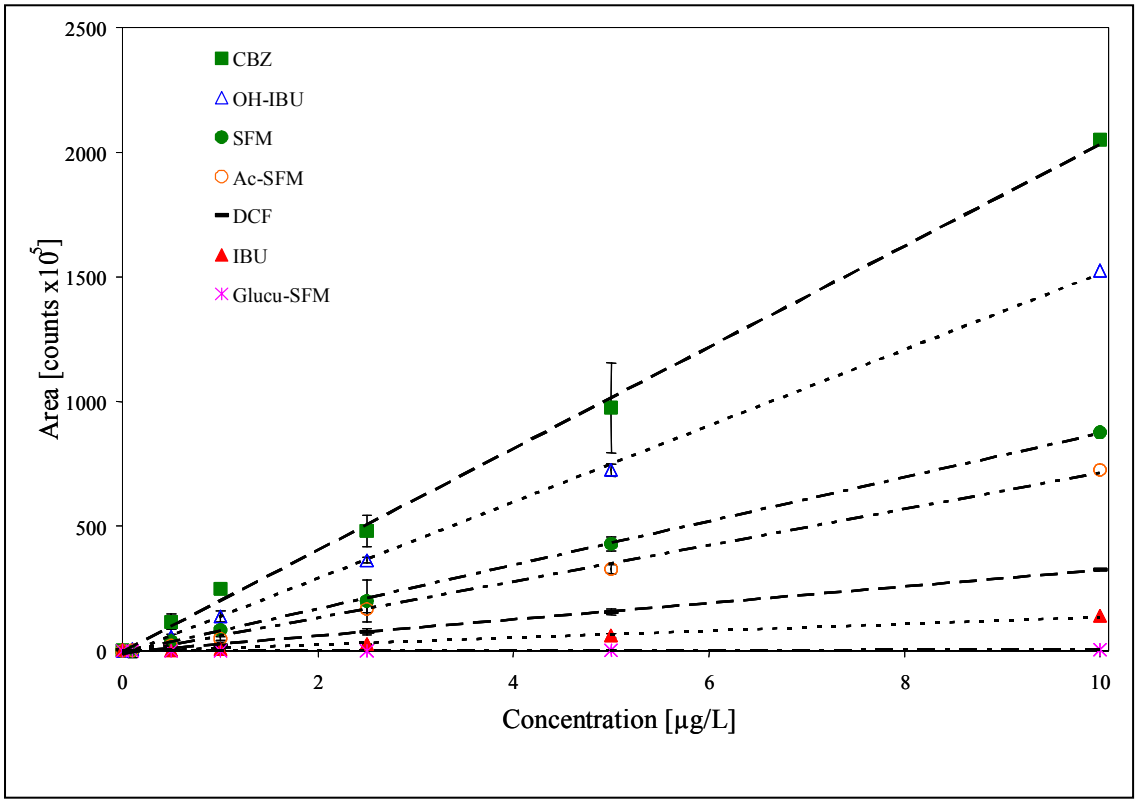


Fig. 3.44: Calibration curves from LC-ESI/MS analysis after SPE (A: 1 L spiked ultrapure water, B: 0.1 L spiked river water; n=3)

3.3.4. Validation of the developed methods

To prove if the developed methods are robust against interferences, parameters having direct influence in the extraction recoveries and signal response, e.g. sample volume, sample concentration and matrix effect were studied.
Three sample volumes (250, 500 and 1000 mL) were chosen to study the enrichment recovery at constant final concentration of a 625 µg/L. To study concentration effects 50, 250 and 1000 ng/L were used at a constant sample volume of 1 L. To ensure the robustness of the developed method, surface water from river Ruhr was used as model for the matrix effect. Detectable concentrations of the selected drugs might be found in real water samples. Therefore, blank values were considered. A control matrix without our analytes as a standard solution was not available. The percentage recovery of the analytes spiked into each type of tested sample was calculated as shown in the (equation 1).

$$\text{Recovery} = \frac{\textit{Area of spiked sample} - \textit{Area of unspiked sample}}{\textit{Area of non enriched standard}} \times 100 \qquad \text{(Equation 1)}$$

As shown in (Table 3.8 – 3.9), we can recognise that neither an increasing sample volume nor the sample concentration had a noticeable influence in extraction recoveries for both methods. However, the response factors obtained from test solutions and matrix loaded samples differ significantly for both methods especially in LC-ESI/MS analysis for some analytes (Table 3.10). The recoveries of the spiked analytes from different volumes (250-1000 mL) and concentrations (50-1000 ng/L) by means of GC/MS were ranged between 91-123 % and 71-113 % respectively, while in LC-ESI/MS were 57-90 % and 47-94 %. For surface water the recoveries of the spiked analytes from different concentrations (2.5-10 µg/L) were between 91-139 % based on GC/MS and 44-81 % for LC-ESI/MS, but for Glucu-SFM the recovery was only about 10 % (Table 3.10). At lower concentration (0.5 µg/L), the analytes showed lower recoveries values for most of the analytes as shown in Table 3.10. In contrast to the standard solution in ultrapure water a loaded matrix had a significant influence on Glucu-SFM. The reason is that the glucuronide moiety hydrolysed in the matrix as proved in chapter 3.4.

In GC/MS, in some cases the extraction recovery values were 10-20 % higher than the non-enriched standard solutions. A feasible interpretation may be that these compounds are present in the original surface water in very low concentrations, which could not be detected before spiking as reported by Sacher F. et al. [82]. In fact, the matrix did not show any influence and that might be attributed to the high molecular compounds which are retarded in the GC injector. Moreover, the ionization technique used prevents the formation of any artifact during the ionization process in comparison with other techniques.

In LC-ESI/MS method, the matrix showed significant influence in the recovery values (Table 3.10). These losses were caused by matrix impurities, which either reduced the sorption efficiencies or led to signal suppression in the ESI interface, which is assumed to result from the competition of the analyte ions and matrix components for access to the droplet surface for the gas-phase emission [83].

The comparison of the recovery values of enriched standard solutions using both methods showed higher recovery values in GC/MS especially for IBU and OH-IBU as demonstrated in Table 3.8 – 3.9. Theses results prove that the ionisation in the ESI is responsible for the loss in the recovery and not to the sorption efficiencies of the SPE material.

Table 3.8: Influence of sample volumes in the extraction recoveries (R) of selected analytes (625 µg/L; final concentration, 1 L sample volume, n = 2)

Analyte	250 mL				500 mL				1000 mL			
	GC/MS		LC/MS		GC/MS		LC/MS		GC/MS		LC/MS	
	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]
Glucu-SFM	-	-	65	*11*	-	-	60	*4*	-	-	58	*3*
SFM	-	-	73	*10*	-	-	71	*10*	-	-	72	*8*
Ac-SFM	-	-	90	*6*	-	-	69	*13*	-	-	76	*4*
OH-IBU	119	*2*	88	*4*	110	*7*	87	*2*	111	*3*	70	*7*
CBZ	123	*3*	81	*4*	115	*8*	72	*11*	114	*2*	82	*1*
DCF	96	*2*	82	*1*	91	*9*	81	*1*	92	*0*	82	*7*
IBU	116	*3*	66	*0*	93	*0*	66	*2*	100	*9*	57	*6*

Table 3.9: Influence of concentrations in the extraction recoveries (R) of selected analytes (625 µg/L; final concentration, 1 L sample volume, n = 2)

Analyte	50 ng/L				250 ng/L				1000 ng/L			
	GC/MS		LC/MS		GC/MS		LC/MS		GC/MS		LC/MS	
	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]
Glucu-SFM	-	-	47	*6*	-	-	58	*3*	-	-	68	*9*
SFM	-	-	60	*8*	-	-	72	*8*	-	-	69	*13*
Ac-SFM	-	-	77	*4*	-	-	76	*4*	-	-	81	*5*
OH-IBU	94	*6*	71	*8*	111	*3*	69	*7*	111	*3*	64	*10*
CBZ	95	*3*	80	*5*	113	*2*	81	*1*	106	*3*	85	*8*
DCF	75	*2*	94	*4*	90	*0*	82	*7*	88	*3*	84	*7*
IBU	71	*25*	63	*4*	99	*9*	57	*3*	79	*0*	60	*17*

Table 3.10: Influence of surface water matrix from the river Ruhr in the extraction recoveries (R) of target analytes (100 mL sample volume, enrichment factors 250 and n=3)

Analyte	500 ng/L				2500 ng/L				10000 ng/L			
	GC/MS		LC/MS		GC/MS		LC/MS		GC/MS		LC/MS	
	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]	R [%]	RSD [%]
Glucu-SFM	-	-	9	*19*	-	-	8	*7*	-	-	11	*9*
SFM	-	-	39	*3*	-	-	44	*3*	-	-	46	*3*
Ac-SFM	-	-	63	*9*	-	-	74	*5*	-	-	76	*2*
OH-IBU	132	*2*	41	*11*	139	*2*	57	*6*	129	*4*	61	*2*
CBZ	132	*3*	51	*10*	120	*3*	54	*2*	109	*5*	78	*8*
DCF	98	*2*	37	*19*	110	*7*	81	*13*	97	*4*	78	*3*
IBU	118	*6*	21	*2*	123	*5*	50	*4*	91	*3*	58	*5*

3.3.5. Comparison between the methods developed

GC-MS and LC-ESI/MS techniques were compared both to find out the most suited method concerning chromatographic (peak shape, resolution, etc.), mass spectrometric (efficiency, sensitivity, etc.) properties and to explore the potential for a multiresidual method.

GC/MS method separated the target analytes with high resolution. In LC-ESI/MS, the drugs and their metabolites can be separated in a single run. But because of the wide pK_a range of the analytes the pH-value of the LC eluent and the sample has a great influence on retention times and separation characteristics and claims much attention.

The MS/MS technique in LC-ESI/MS method provides better sensitivities and gives additional information. As well in GC/MS, SIM gives high sensitivities.

When comparing the recovery values from the both methods, we could discriminate the influence of the matrix on the ionization efficiency in LC-ESI/MS.

The GC/MS measurements reproducibility was represented with lower RSD's. On the other hand, acceptable reproducibility was achieved for LC-ESI/MS due to the absence of internal standard correction.

GC/MS method provides a better accuracy for all investigated analytes except SFM and its metabolites.

Both methods showed very good linearity ($r^2 > 0.97$) in most cases and have a LOQs from 3 to 10 ng/L for all compounds, except for SFM and its metabolites in GC method.

3.3.6. Applications of the developed methods for a real surface water sample

The developed methods were tested for applicability to real water samples by measuring surface water samples from river Ruhr. Concentrations of the selected drugs and some of their metabolites are listed in (Table 3.11). The table demonstrates that GC/MS is best for the determination of IBU, OH-IBU, DCF, while LC-ESI/MS is best for SFM and Ac-SFM; CBZ can be determined with both methods. Quantification on both methods was based on a calibration curves build with the same matrix as described in the previous sections.

Four drugs and some of their main metabolites could be identified in relative high concentrations. That is not surprisingly when considering that the effluent of a hospital enters the river about to the place of sampling. Using GC/MS the concentrations in river water ranged DCF, IBU, OH-IBU and CBZ from 107 to 249 ng/L. The results for the two SFM analytes - measured by LC-ESI/MS – were 229 ng/L for SFM and 316 ng/L for Ac-SFM, while the concentration of CBZ was 290 ng/L.

In comparison with diverse studies for drugs concentrations in German rivers [2], these magnitudes are mentioned for drugs detected at similar concentrations. Glucu-SFM was not detected due to possible cleavage of the glucuronides in surface water.

Table 3.11: Concentration (ng/L) of the selected pharmaceuticals and some of their metabolites in surface water (Ruhr river, Germany on 04.06.2004)

Analyte	GC/MS method [ng/L]	**RSD** [%]	LC-ESI/MS method [ng/L]	**RSD** [%]
Glucu-SFM	-	-	n.d.	-
SFM	-	-	229	*51*
Ac-SFM	-	-	316	*46*
OH-IBU	249	*10*	-	-
CBZ	241	*13*	290	*39*
DCF	107	*25*	-	-
IBU	169	*31*	-	-

- : method non-suitable
n.d. : not detected

3.3.7. Conclusion

Two analytical methods based on a pre-concentration step by solid phase extraction followed by GC/MS and LC-ESI/MS were developed for simultaneous determination of traces of target drugs and their main metabolites. Derivatization step was necessary prior to GC/MS analysis.

After examination a varieties of sorbent materials, Oasis HLB showed the best extraction recoveries for all target analytes. The analytes concentrations and sample volumes showed nearly no influence in the extraction efficiency.

Due to the elevated polarity of structure moieties of the analytes, the extract has to be derivatize prior to the measurement for GC/MS. Among many reagents, diazomethane was the best reagent for most analytes except CBZ and Diol-CBZ. But that doesn't matter, because CBZ and Diol-CBZ could be analysed without derivatization. The advantage of diazomethane is that there are a few by-products and the molecular weight of the derivatives increased slightly.

Regarding the chromatography conditions, the HPLC mobile phase composition and it pH value had tremendous influence on the retention time of acid functionality and ions formation in ESI interface. Therefore, the final method was the best compromise between these parameters.

For more selectivity and specificity, GC/MS method was based on selected ion monitoring, while selected reaction monitoring was used in LC-ESI/MS.

For ultrapure water samples applying the final procedures the recoveries of the spiked analytes at constant sample volume (1 L) and concentration (1 µg/L) were 79-111 % by means of GC/MS and 60-85 % using LC-ESI/MS. For surface water the recoveries were 91-129 % based on GC/MS and with LC-ESI/MS 46-78 % except for Glucu-SFM. The method detection limits were 1-5 ng/L in GC/MS except the SFM and its metabolites and 3-5 ng/L in LC-ESI/MS for all analytes.

The GC/MS method showed high linearity, high resolution, good precision, low DLs and nearly no matrix influence. The only disadvantage of this method is that SFM, Ac-SFM and Glucu-SFM are not suitable for quantifications. On the other hand also the LC-ESI/MS method showed high linearity, good resolution, acceptable precision, low DLs and tangible matrix dependence as results of the signal suppression, caused by high amounts of organic and inorganic ions in the sample.

As a consequence, the determination of DCF, IBU and OH-IBU were preferred by GC/MS, whereas, SFM, Ac-SFM and Glucu-SFM were preferred by LC-ESI/MS. CBZ could be determined by the both methods.

The developed methods were verified by analysis of surface water samples from the river Ruhr. All the target analytes were detected except Glucu-SFM. Using GC/MS the concentration of DFC, IBU, OH-IBU and CBZ ranged from 107 to 249 ng/L, while in LC-ESI/MS the concentration of SFM, Ac-SFM and CBZ ranged from 229 to 316 ng/L.

3.4. Investigation of the biodegradation of drugs in different model systems

There is still a lack of data on the fate, elimination mechanisms and efficiencies in sewage and drinking water treatment especially for the metabolites and transformation products. In addition, the understanding of physical, biological and chemical behavior in the aquatic system such as adsorption, degradation and hydrolysis is of great importance as pathways for natural treatment. The fate of the most selected drugs is not well understood yet in the aquatic environment. Up to now only a few data's are available about the fate of the human metabolites of CBZ, DCF and SFM in the aquatic environment.

The analytical developed methods were tested to study the behavior fate of our target analytes in batch and column experiments. Such experiments give an important knowledge about the degradation behaviour of the pharmaceuticals in the dependence of the amount of the dissolved oxygen concentration, pH-values or microbial populations.

The experiments are carried out with relatively higher concentration (1 mg/L) to simplify the detection of any possible transformation products. The described LC-UV developed methods in section 3.2.1 were used in order to monitor the behaviour of the analytes concentration during the period of test time. Besides that, the attempt was started to identify the single degradation products by means of LC-ESI/MS and GC/MS. For that purpose an enrichment procedure as described in section 3.3.1 was applied. These results are discussed in section 3.4.4. The aim from the further mass spectrometry investigation was qualitative aspects.

3.4.1. Purity investigation of the standard drug materials

Besides that the analysis is complicated by a lot of compounds similar to the drugs, which have their origin in the drug preparation and are still present in the applied products. Therefore, the purity of the commercially available standard compounds and synthesised metabolites were justified in the preliminary stage of this study in order to discriminate between the presence of trace products in the original starting materials and the products formed during the biodegradation processes.
Using GC/MS, traces of the OH-IBU could be identified in **IBU** (Fig. 3.45) and the synthesized **OH-IBU** showed traces from IBU, IBU-Me, and CA-HA (R_t: 16.05) (Fig. 3.46). The LC/MS method verified these results (Fig. 3.47).

By the analysis of GC/MS, **CBZ** showed traces of Diol-CBZ (R_t: 21.08), CBZ-ME (R_t: 21.47), and a signal at R_t: 18.29 can probably be assessed to CBZ-OH and at R_t: 19.88 to EP-CBZ (Fig. 3.48). Both substances cannot be clearly identified. LC/MS verified the presence of Diol-CBZ (R_t: 8.7), CBZ-OH and/or EP-CBZ (R_t: 11.04) and CBZ-Me (R_t: 11.47) (Fig. 3.49).

Using GC/MS, **DCF** showed traces of OH-DCF (R_t: 26.03), 8-CCA (R_t: 23.27) and indole (R_t: 22.0) (Fig. 3.50). LC/MS verified the presence of traces from OH-DCF (R_t: 14.81) and 8-CCA (R_t: 17.44) (Fig. 3.51).

Using GC/MS, **SFM** showed traces of Acetamide-N-Phenyl (Rt: 10.73), 4-Aminobenzene-sulfonamide (Rt: 20.99) and methylated SFM (Rt: 24.75). LC/MS verified the results (Fig. 3.52). Using GC/MS, the synthesized **Ac-SFM** showed traces from Acetamide-N-Phenyl, 4-Aminobenzensulfonamide, SFM and methylated Ac-SFM. LC/MS verified the presence of methylated Ac-SFM (Fig. 3.53). The LC/MS chromatogram from synthesized **Glucu-SFM** showed the presence of mono-, di- and tri-methylated Glucu-SFM as synthesised products (Fig. 3.54).

The source of the methylated products could be by-products of synthesis or contaminants of the starting compounds, is not yet clear. It could be recognised based on LC/MS and GC/MS analysis of the standard solutions presence of additional products from synthesis, which must be considered in the further study.

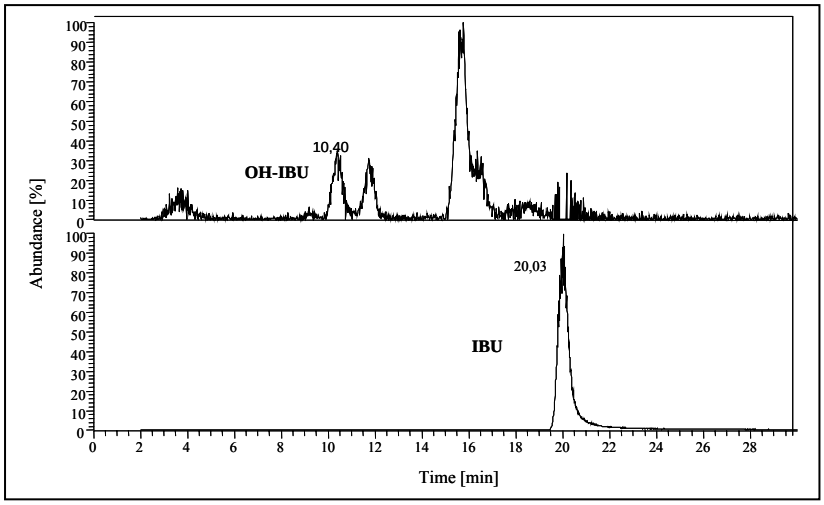


Fig. 3.45: Extracted mass chromatograms from LC/MS analysis of the standard IBU solution

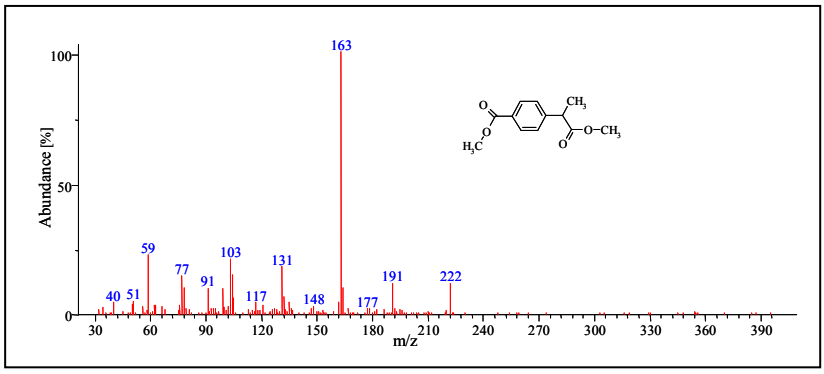


Fig. 3.46: Electron ionization mass spectra of the diazomethane derivative of the CA-HA

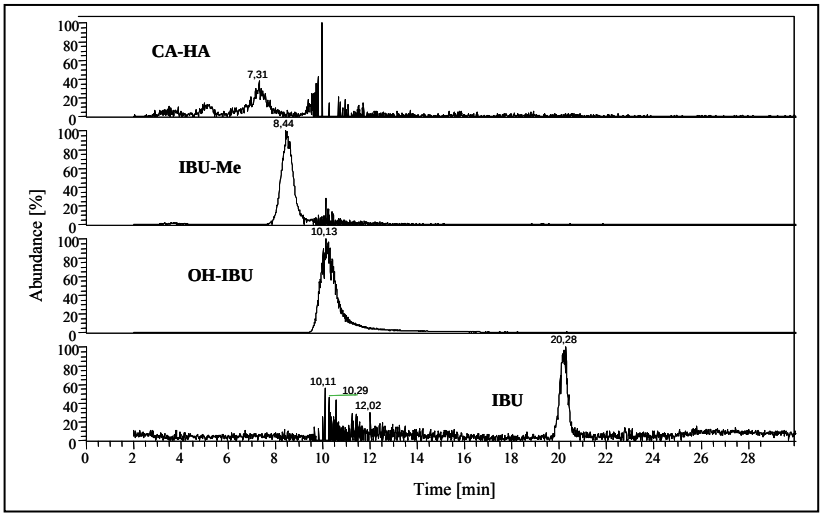


Fig. 3.47: Extracted mass chromatograms from LC/MS analysis of the synthesised OH-IBU solution

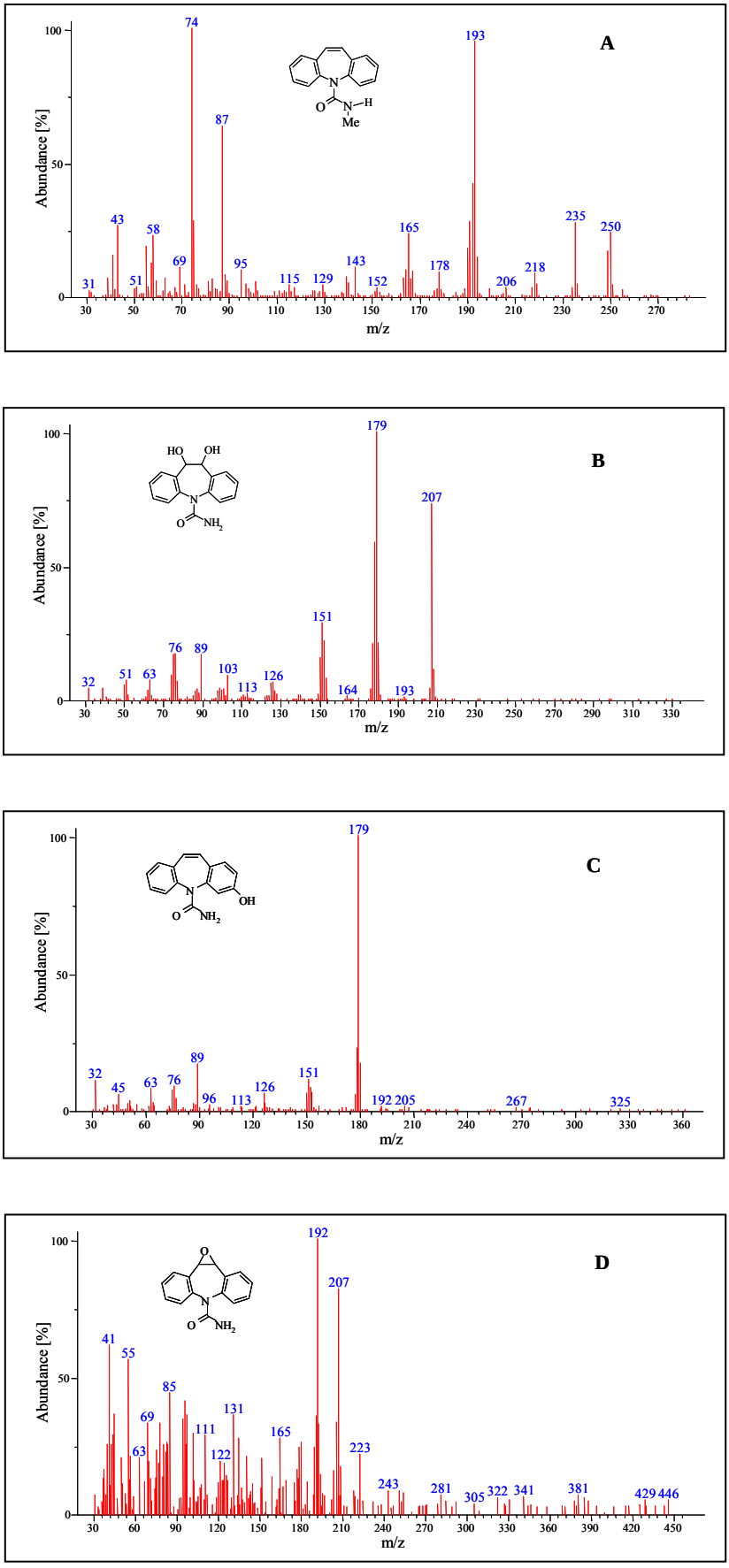


Fig. 3.48: Electron ionization mass spectra of the CBZ-Me (A), Diol-CBZ (B), CBZ-OH (C) and EP-CBZ(D)

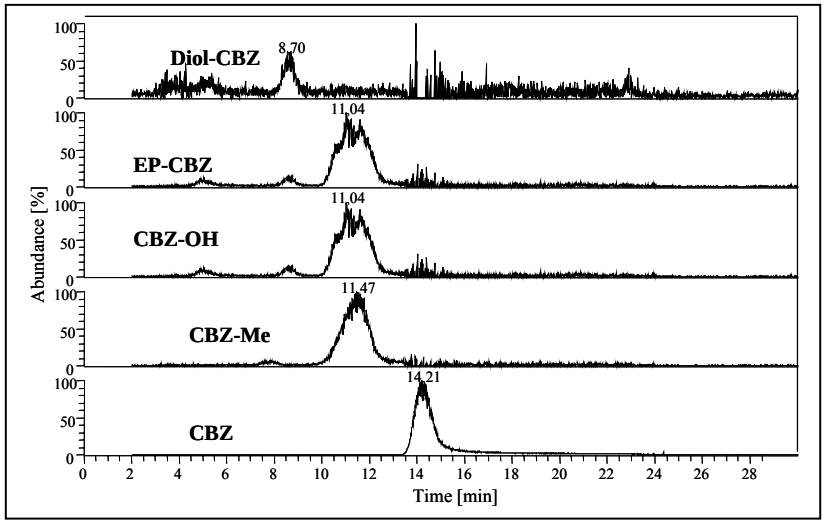


Fig. 3.49: Extracted mass chromatograms from LC/MS analysis of the standard CBZ solution

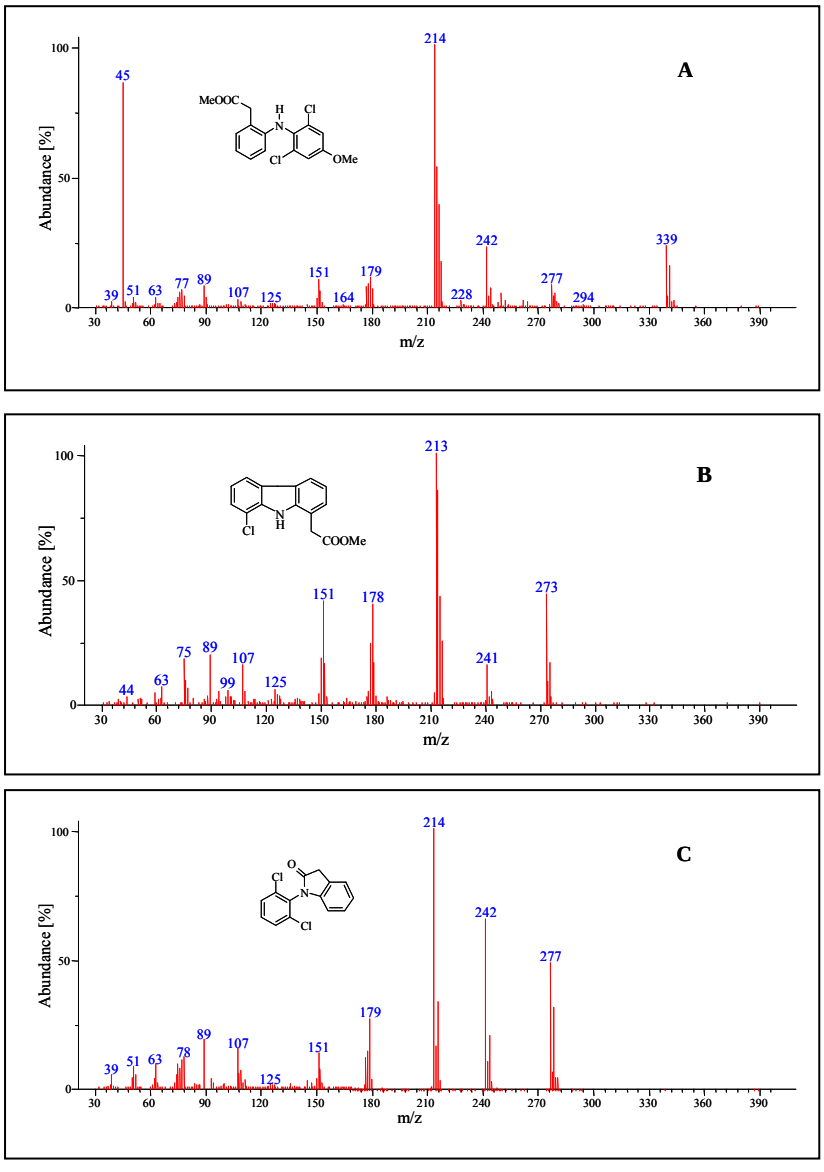


Fig. 3.50: Electron ionization mass spectra of the diazomethane derivative of the OH-DCF (A), 8-CCA (B) and Indole (C)

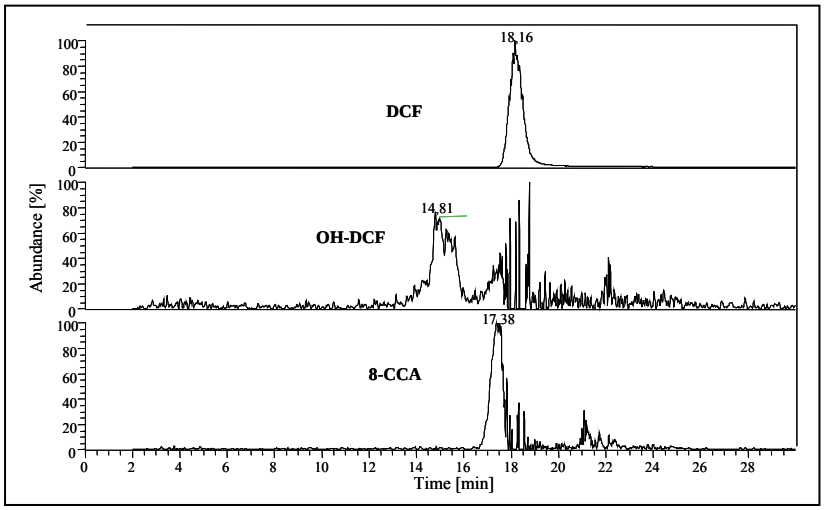


Fig. 3.51: Extracted mass chromatograms from LC/MS analysis of the standard DCF solution

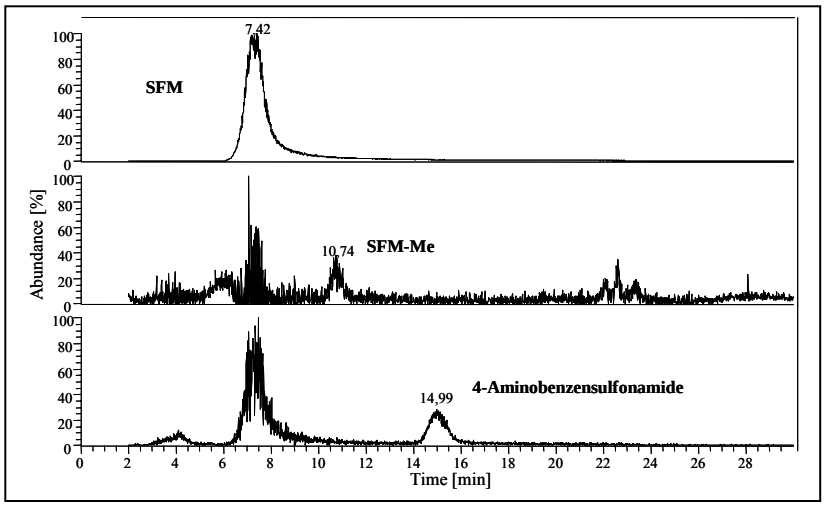


Fig. 3.52: Extracted mass chromatograms from LC/MS analysis of the standard SFM solution

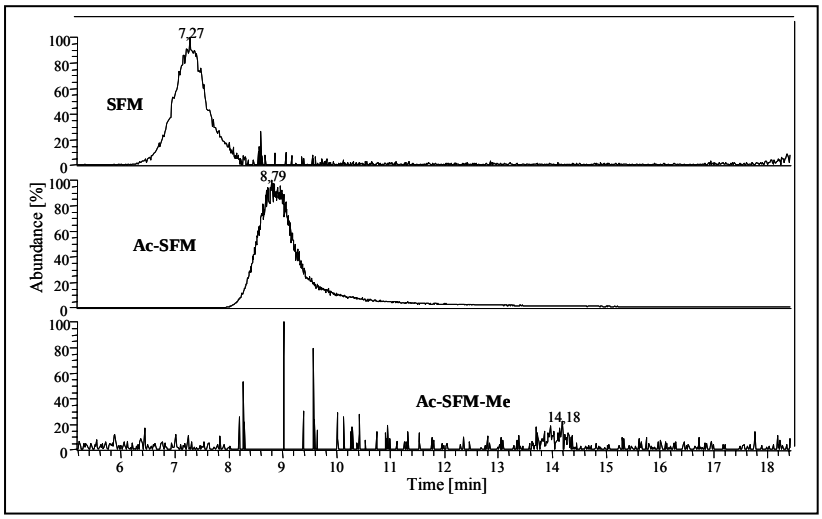


Fig. 3.53: Extracted mass chromatograms from LC/MS analysis of the synthesised Ac-SFM solution

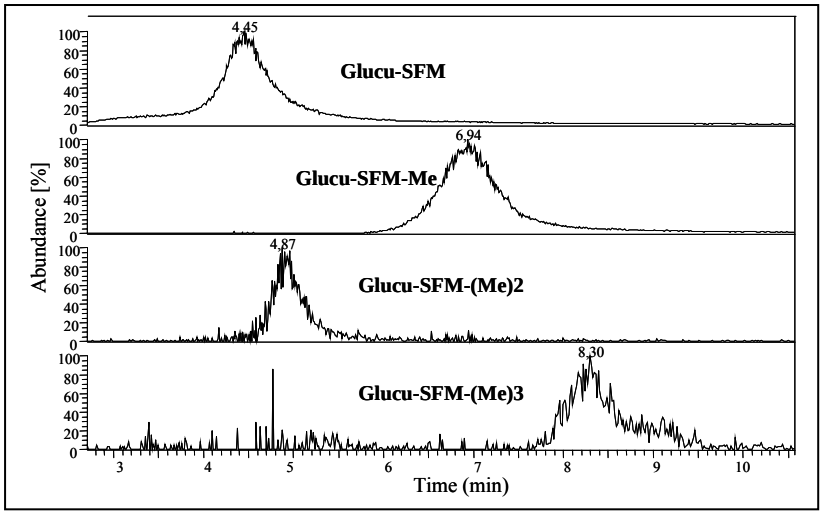


Fig. 3.54: Extracted mass chromatograms from LC/MS analysis of the synthesised Glucu-SFM solution

3.4.2. Batch experiments

The MITI-tests, following the OECD method (described in chapter 5.4), examined the degradation of the model substances CBZ, DCF, IBU and SFM in ground water (GW) and surface water from the river Ruhr affected by the run off of a wastewater sewage plant, but further on, to simplify matters, only called surface water mix (SWM). The experiments were prepared parallel in sterile and unsterile water in order to be able to differentiate between microbiological and chemical conversions.

The composition of the batch solution contains 1 mg/L of the 4 analytes each, dissolved in a matrix of culture medium, which contains 16 % ground water or the surface water mix. The culture medium composition was prepared as described in chapter 5.4.

3.4.2.1. The behaviour of the target drugs

Sterile waters
The experiments under sterile conditions showed no decrease in concentration of the active substances within a month for both ground water and surface water mix (Fig. 3.55). Because of the distinct results with the target compounds the experiments in sterile waters were dropped.

Unsterile waters
A strong loss in concentration could be observed for SFM in both batches (Ground water, surface water mix) after one month. Besides that, in the surface water mix occurred a dramatic decrease of concentration of IBU after 25 days as shown in the (Fig. 3.56).

Two months later the four tests were measured again to get additional information. In surface water mix IBU and SFM showed a complete degradation process, while the concentration of SFM in ground water batch decreased to 26 %, and within the next 2 weeks it was degraded totally. The concentration of IBU in the ground batch was constant; the degradation of IBU started after 6 months, the concentration decreased up to 33 % within 35 days. The other analytes showed constant concentrations in both batches as shown in Fig. 3.56.
In both Fig. 3.55 and Fig. 3.56, there is little gap in the graph after 30 days. The reason is a necessary exchange of the analytical column. Repeating the test under unsterile surface water

mix conditions, the result validated the degradation of IBU, which was significantly dismantled within two weeks. On the other hand the degradation of SFM started two months later and was completed after two and a half month (Fig. 3.57). The other analytes showed similar behaviour as shown in the previous tests.

The difference between the two tests may be caused by the differences of water ingredients of the surface water, taken at different times from the river. In the conventional water treatment plants the wastewater storage period normally is very short. Therefore, the time isn't long enough to degrading these substances significantly. An exception is IBU, which was degraded depending on the seasonal water ingredient.

3.4.2.2. The behaviour of the synthesised metabolites

Applying the same approach of batch experiments, the synthesised metabolites Glucu-SFM, Ac-SFM, OH-IBU and Diol-CBZ were investigated in order to compare the behaviour of degradation with the parent analytes in both water types.

Surface water mix
The results showed that in this water the metabolites Glucu-SFM, OH-IBU, and Ac-SFM degraded significantly under batch conditions with a different speed (Fig. 3.58); the signal of Ac-SFM has disappeared completely after 2 weeks, Glucu-SFM after 5 weeks and OH-IBU after 8 weeks. Only Diol-CBZ kept its concentration value.

Ground water
All the investigated metabolites showed high stability under such water batch conditions (Fig. 3.58).

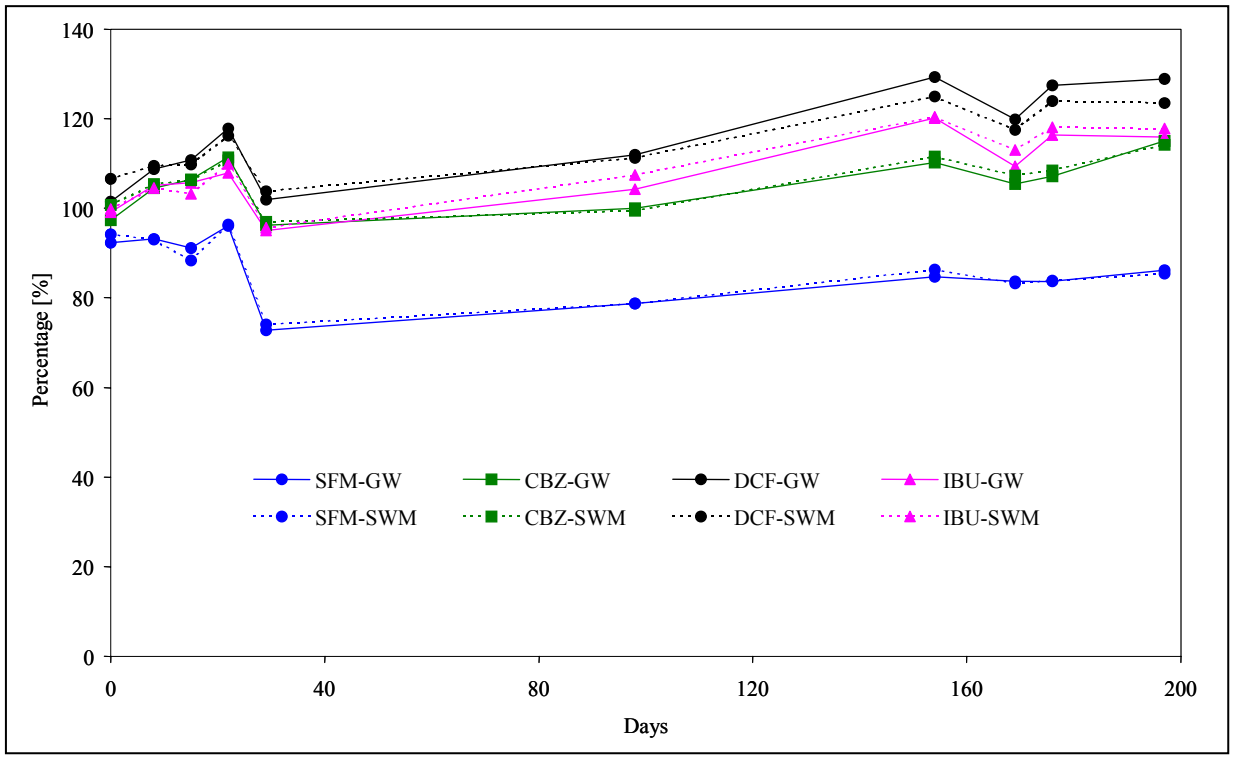


Fig. 3.55: The influence of sterile batch conditions on the target pharmaceuticals (GW: ground water, SWM: surface water mix)

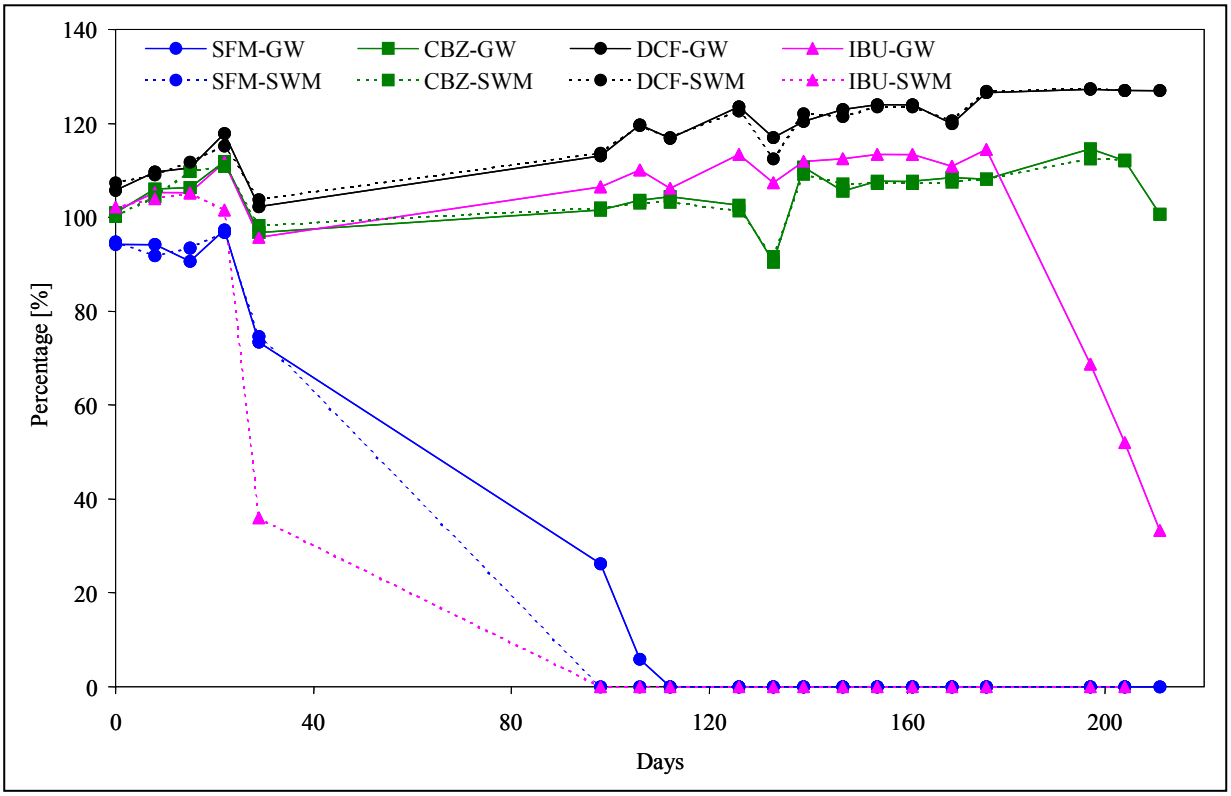


Fig. 3.56: The influence of unsterile batch conditions on the target pharmaceuticals (GW: ground water, SWM: surface water mix)

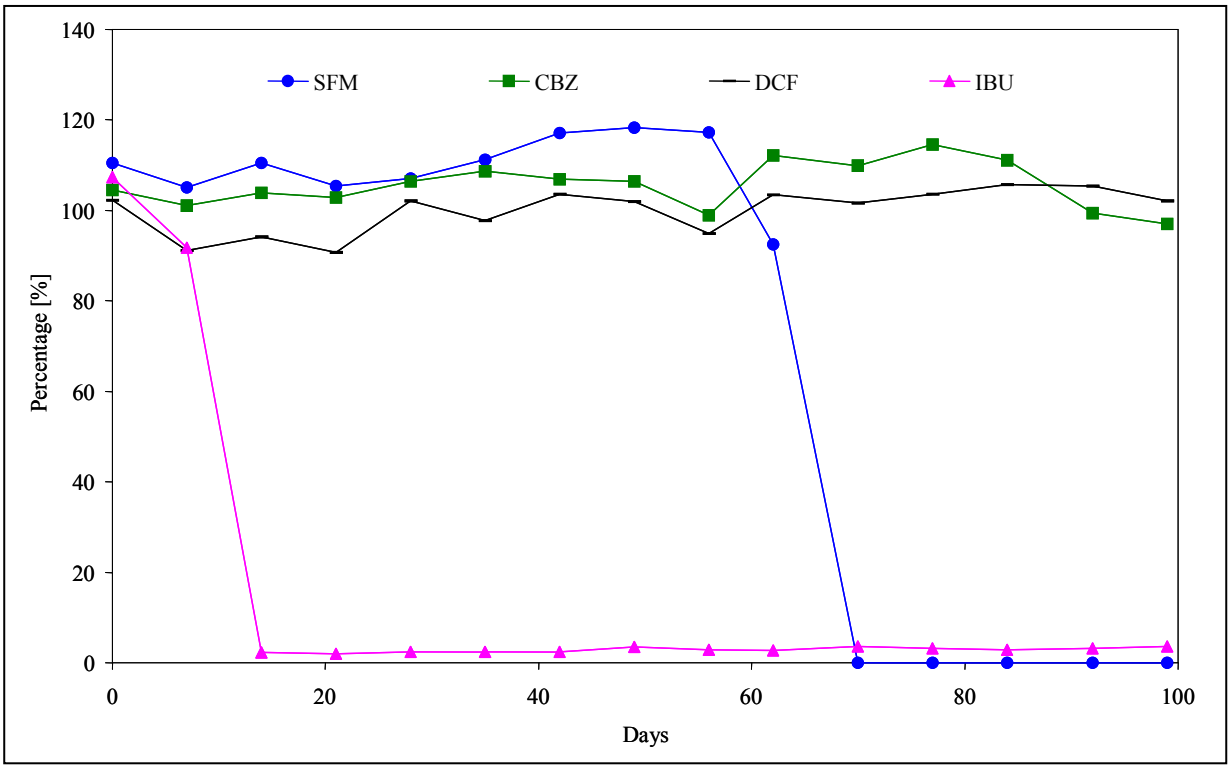


Fig. 3.57: The influence of unsterile SWM batch condition on the target pharmaceuticals (trial 2), (GW: ground water, SWM: surface water mix)

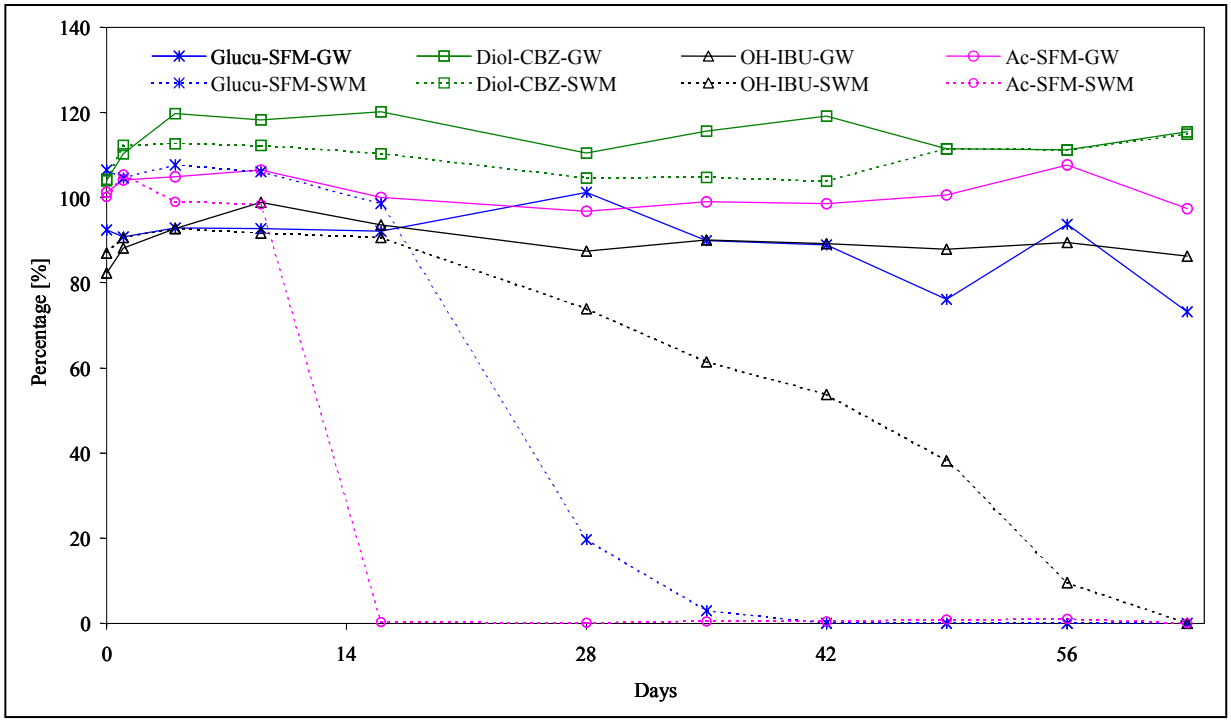


Fig. 3.58: The behaviour of the investigated metabolites under unsterile batch conditions (GW: ground water, SWM: surface water mix)

3.4.3. Column experiments

The column experiments were performed at the pilot plant station by the *Institute for Water Research* in Schwerte-Geisecke. The columns set up are shown in (Fig. 3.59) and the technical dates are given in section 5.4. The interpretation of the tests samples were done at the Institute For Analytical Sciences in Dortmund.

The goal of the column experiments was the semi-natural simulation of the rinsing water through the sediment passage. The columns were filled with Rhine sand on which a biofilm was grown by feeding the columns with ground or surface waters from the river Ruhr.

The columns were operated at the following conditions:

- Column I: operated with natural aerobic ground water, which is relative poor in microorganisms
- Column II: operated by conditioning with surface water from river Ruhr
- Column III: operated by conditioning with surface water from river Ruhr in addition to potassium chloride (400 mg/L)
- Column IV: repetition of the column II under the same condition (flow path)
- Column V: repetition of the column II, but the test solution was cycled in a closed circuit (closed circuit)

The content of the microorganisms in surface water was characterised by a comparison with ground water (chapter 5.4). After stabilization of the hydraulic, chemical and biological conditions, surface and ground waters were spiked with 100 µg/L each of the four analytes and sucked through the columns at a flow rate of 1.6 L/h. The period of the test took four weeks continuously.

3.4.3.1. The behaviour of the drugs

The results of this investigation (column I - III) showed that only the concentration of IBU decreased significantly in the columns II and III. The other drugs showed high stability under the adapted conditions. In column I the concentration kept constant.

The sample measurements from column IV showed constant concentration levels during the test time (Fig. 3.60A). Against that, after the adaptation phase of the first week, column V showed a retarded decrease in concentration of SFM, DCF and IBU within the first 7 weeks followed then by a strong degradation (Fig. 3.60B). CBZ nearly kept a constant value over the test period.

This is a known effect in biodegradation. The microbes first consume carbon source easy to digest resulting an increasing population. Later on, they attack other carbon sources such as the drugs.

Fig. 3.59: The set-up of the column experiment, carried out at the 'Institute for Water Research' in Schwerte-Geisecke

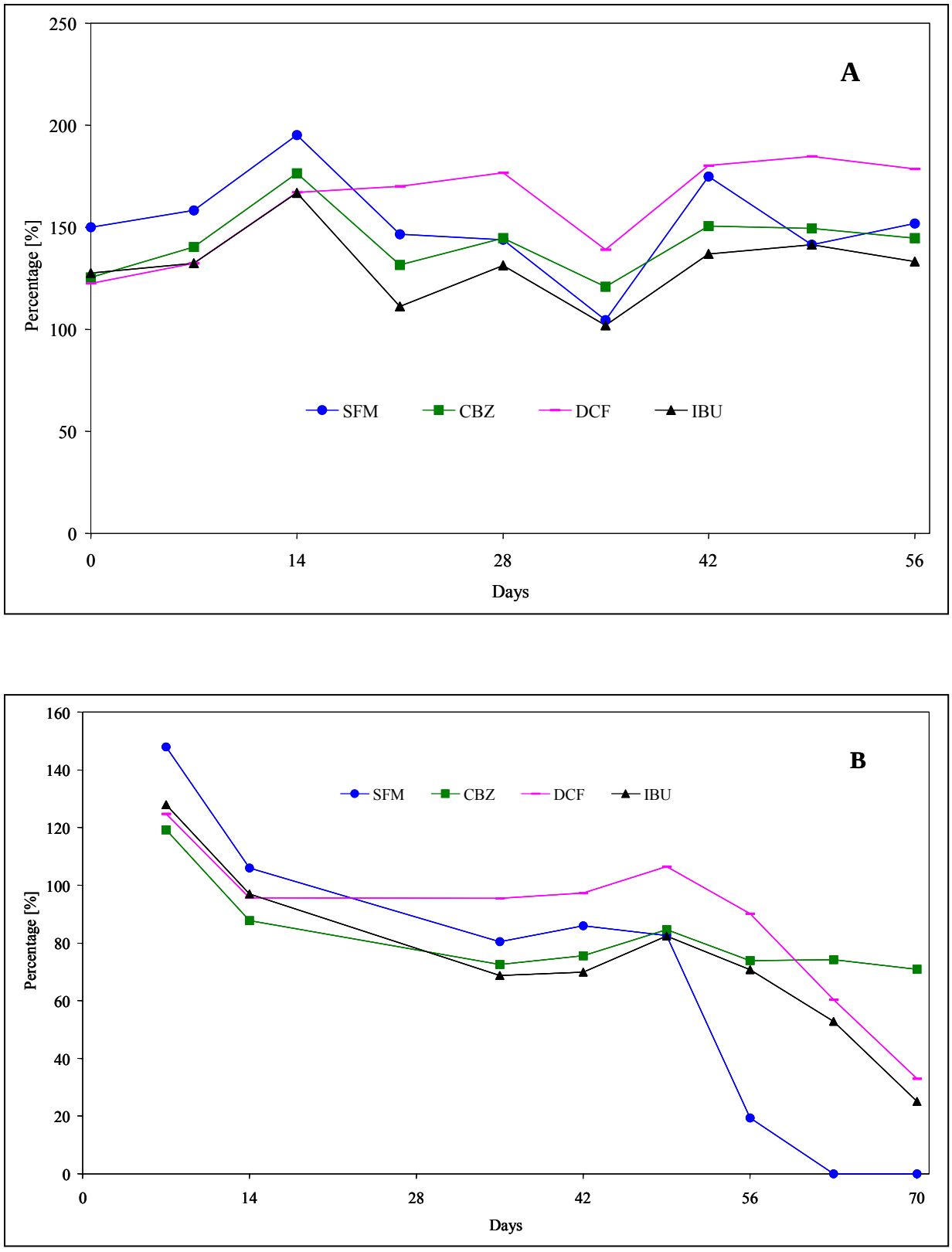


Fig. 3.60: The target pharmaceuticals behaviour under biodegradable conditions
A: Column IV (flow path), **B**: Column V (closed circuit)

3.4.4. Attempts to identify of degradation products

In order to understand the fate of the target drugs and their synthesised metabolites, samples from the batch and column tests were successively collected and analysed with LC- and GC/MS according to the methods developed in chapter 3.3.

3.4.4.1. Degradation products of the target drugs under batch conditions

Because no degradation could be observed in sterile waters, the investigation was concentrated only on unsterile waters.

Under such batch conditions (ground water, surface water mix) the concentration of IBU and SFM decreased significantly as mentioned in section 3.4.2.

For IBU the results were very odd; while in ground water the formation of OH-IBU (Fig. 3.28E) and CA-IBU (Fig. 3.61) were detected, in surface water mix no single IBU metabolite could be detected though the signal from IBU disappeared totally. Against that, SFM showed the same behaviour in both waters. SFM is partially transformed to Ac-SFM which itself is then degraded completely (Fig. 3.71). DCF and CBZ didn't show any formations of degradation products under these conditions.

3.4.4.2. Degradation products of the target drugs under column conditions

In the samples of the first three columns no IBU metabolites could be observed. Though column IV ran under the same conditions as Column II now the formation of OH-IBU, CA-IBU and CA-HA could be detected as shown in (Fig. 3.62). The reason might be a much greater biological activity caused by the flood water situation in that time. Column V showed OH-IBU and a very weak signal of CA-IBU. The concentration of OH-IBU in column IV and V increased with time, but on a very different level (Fig. 3.63). The other metabolite concentrations showed a gradual decrease. The behaviour of IBU metabolites is in agreement with the data mentioned in the literature so far [11,35,90].

In contrast to column IV, where no significant degradation of SFM could be detected, Column V showed a significant decrease in the concentration of SFM and an adequate increase in the concentration of Ac-SFM (Fig. 3.64). Finally a similar fate was observed for SFM and Ac-SFM in the column V as we have seen in the batch experiment.

No metabolites were detected for DCF in column IV. On the other hand, the formation of an unknown DCF-artifact in column V could be found at (Rt: 29.0) in the GC/MS chromatogram (Fig. 3.65). Its concentration increased during the test phase (Fig. 3.66). Under the applied conditions CBZ didn't show the formation of any possible metabolites.

3.4.4.3. Degradation products of the synthesised metabolites under batch condition

Samples from the metabolites batch tests were collected after 5 weeks for further verification of the degradation products by GC/MS and LC/MS.

In contrast to surface water mix where the degradation process needed 10 weeks, OH-IBU showed a more retarded degradation in ground water (Fig. 3.67). But none of the expected transformation compounds could be identified.

The investigation of Ac-SFM behaviour in ground water showed a significant decrease in concentration after 12 weeks (Fig. 3.68); at the same time SFM was detected in a very low concentration. This concentration was always present in the synthesised Ac-SFM as a residual contaminant as mentioned in section 3.4.1. In surface water mix Ac-SFM degraded strongly within 2 weeks as showed in Fig. 3.58. An unknown product from surface water mix batch was detected in the GC/MS chromatogram at (Rt: 25.94) (Fig. 3.69), but a further identification is too difficult to predict the resulted product. A control measurement of the derivatized sample by LC/MS confirmed the formation of an unknown compound at (Rt: 16.88) (Fig. 3.70) It seemed to be a dimethylated SFM, as result from the hydrolysis product of Ac-SFM (Fig. 3.71).

When applying Glucu-SFM in the ground water batch a signal for SFM appeared after 60 days with increasing tendency (Fig. 3.72). That might be caused by hydrolysis of the glucuronide moiety. In the surface water mix batch, SFM was also formed from Glucu-SFM within one and a half week (Fig. 3.71). The formation of SFM was verified with GC/MS. Later on, the formed SFM decreased gradually and degraded totally after 8 weeks in surface water mix. Similar to Ac-SFM, the same unknown peak was detected in the GC/MS chromatogram at (Rt: 25.94) in addition to a new unknown product at (Rt: 22.61) (Fig. 3.73) in both batch tests. Both unknowns showed dismantling fate in surface water mix. The derivatized samples were confirmed by LC/MS, the result confirmed the formation of methylated Ac-SFM, mono-, di- and trimethylated SFM (Fig. 3.74). Possibly these unknown compounds in GC/MS are derivative products as a result from derivatization process.

Diol-CBZ showed constant concentration levels in both tests with ground water and surface water mix.

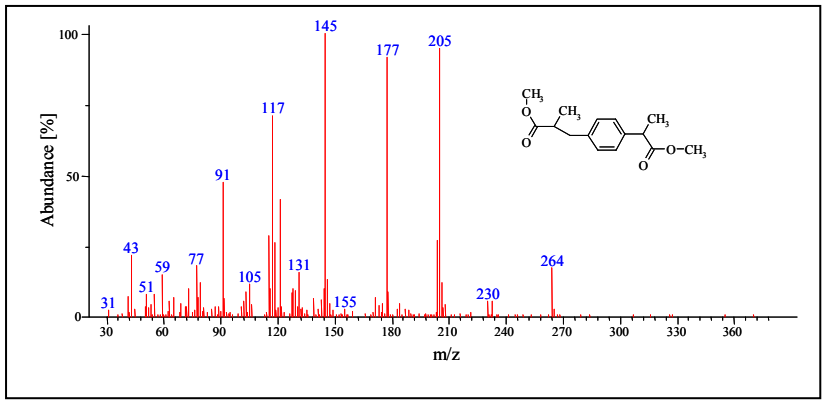


Fig. 3.61: Electron ionization mass spectra of the diazomethane derivative of the IBU metabolite (CA-IBU) formed under batch conditions

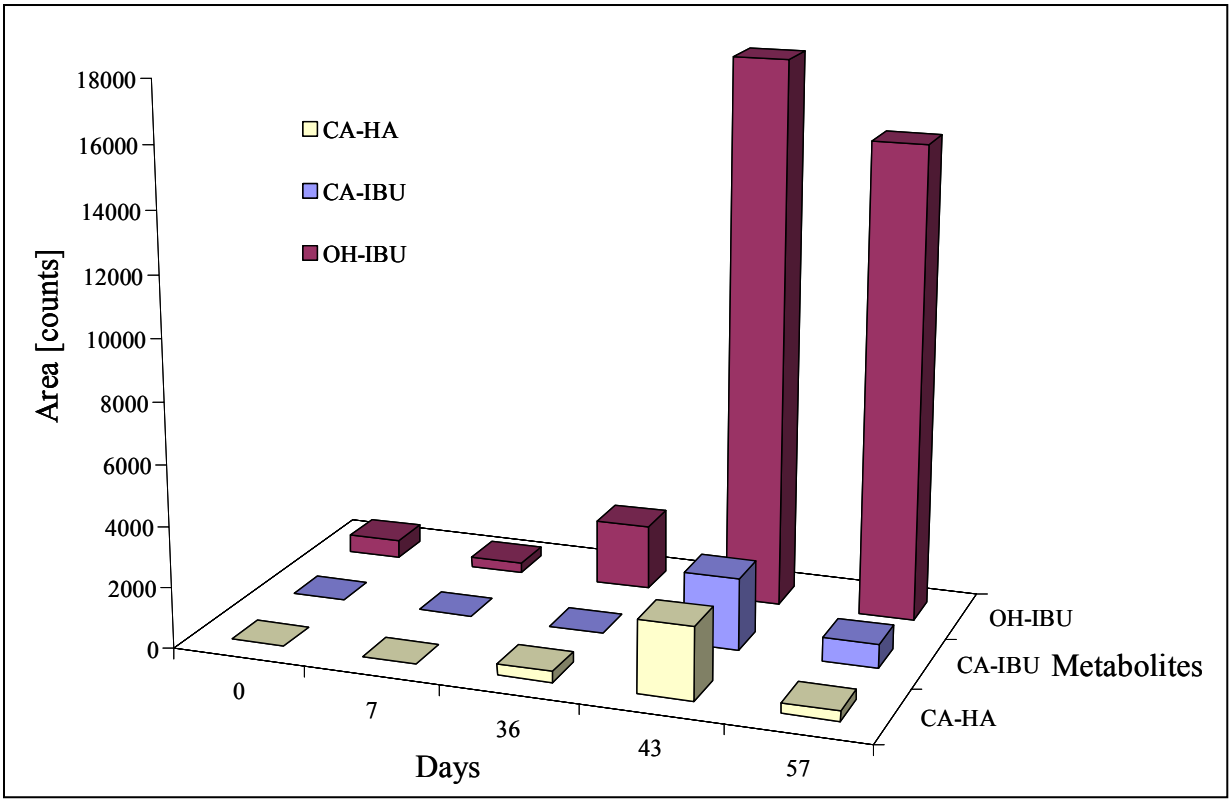


Fig. 3.62: The formation level of IBU metabolites during the flow path column test (GC/MS)

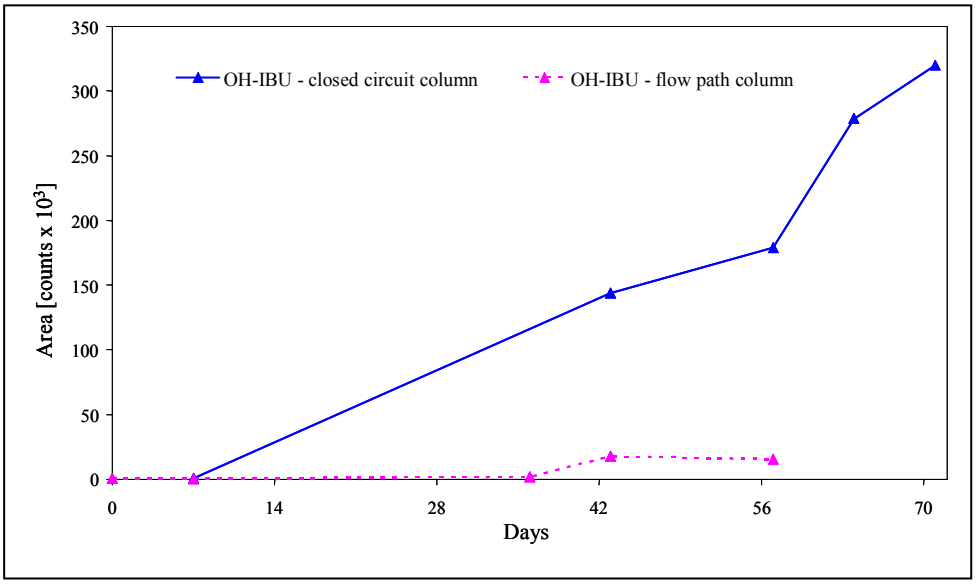
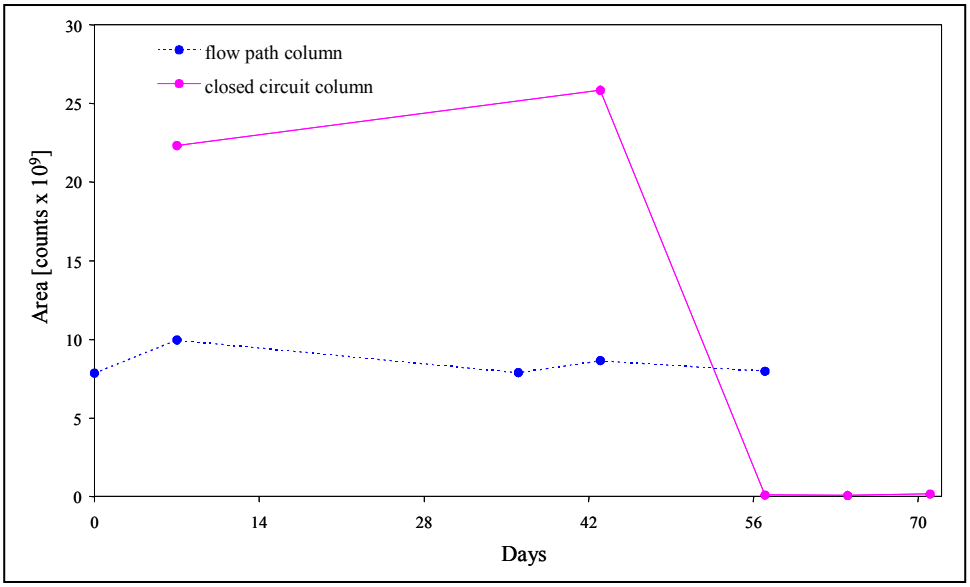


Fig. 3.63: The formation level of IBU-metabolites during the column tests (GC/MS)

A)



B)

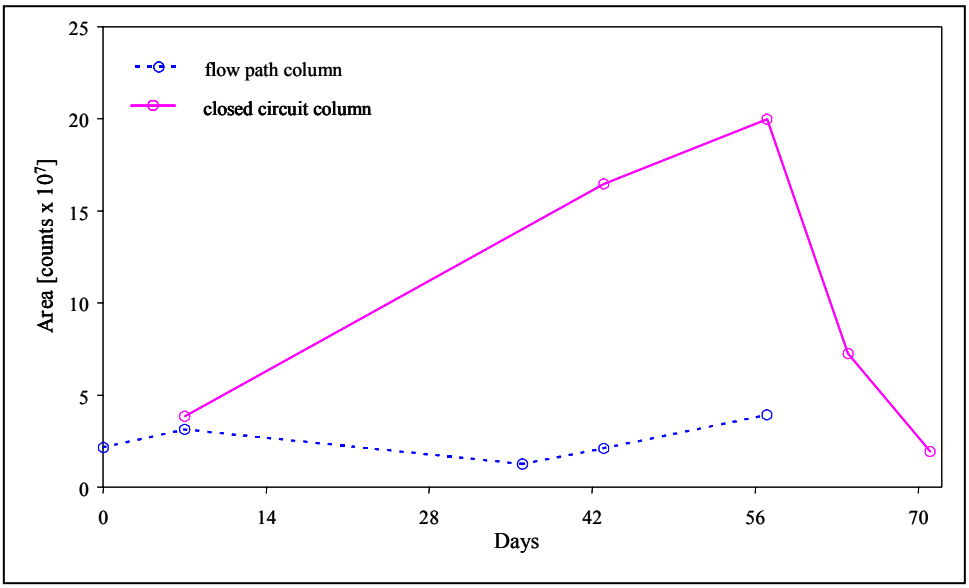


Fig. 3.64: The transformation behaviour of SFM (A) and the formation level of Ac-SFM (B) during the column tests (LC/MS)

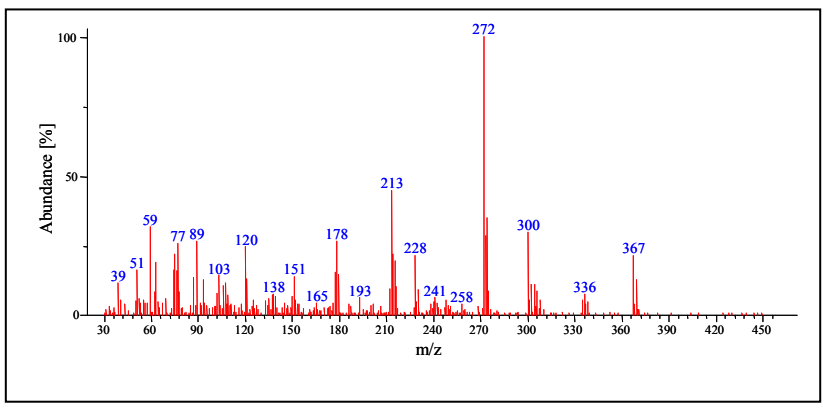


Fig. 3.65: Electron ionization mass spectra of the diazomethane derivative of an unknown DCF-artifact (?) formed during closed circuit column test

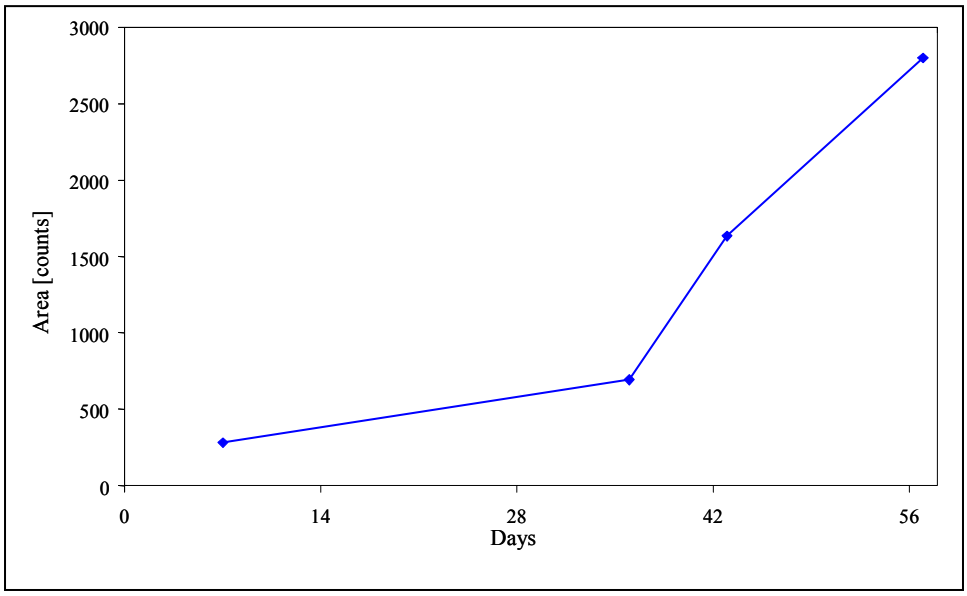


Fig. 3.66: Formation of an unknown DCF-artifact (?) during the closed circuit column test (GC/MS)

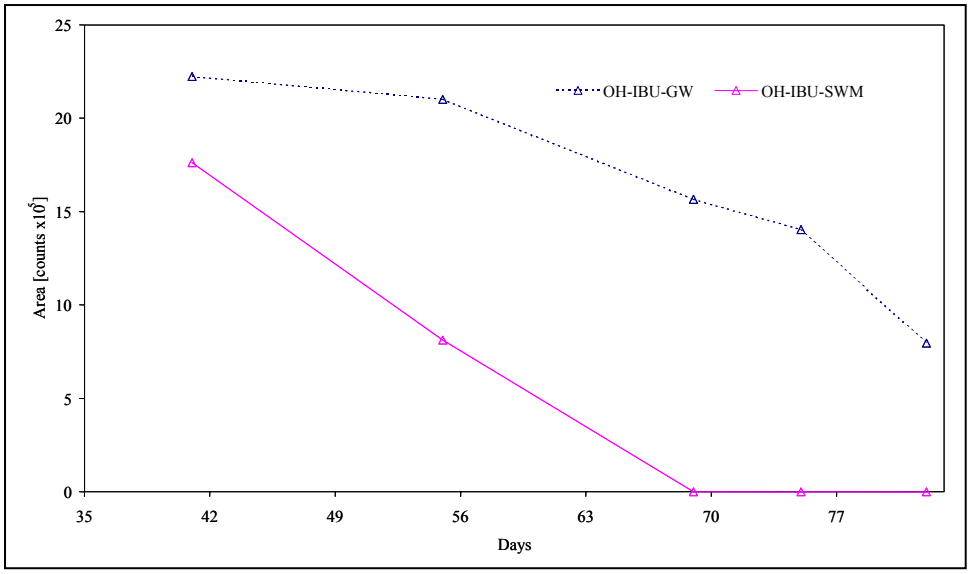


Fig. 3.67: OH-IBU metabolite behaviour under batch conditions (GC/MS) (GW: ground water, SWM: surface water mix)

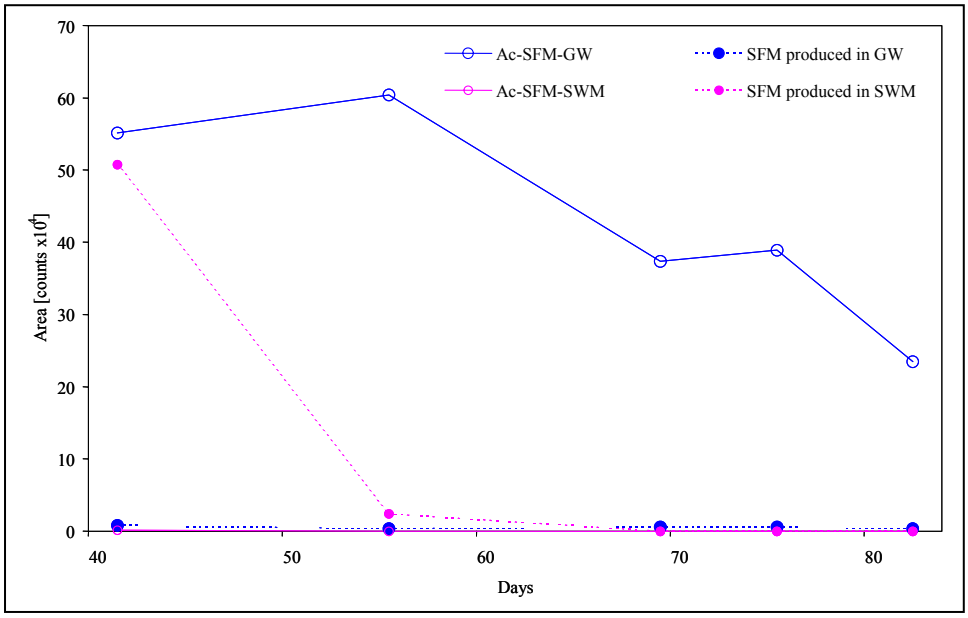


Fig. 3.68: Ac-SFM metabolite behaviour and the formation of SFM under batch conditions (GC/MS), (GW: ground water, SWM: surface water mix)

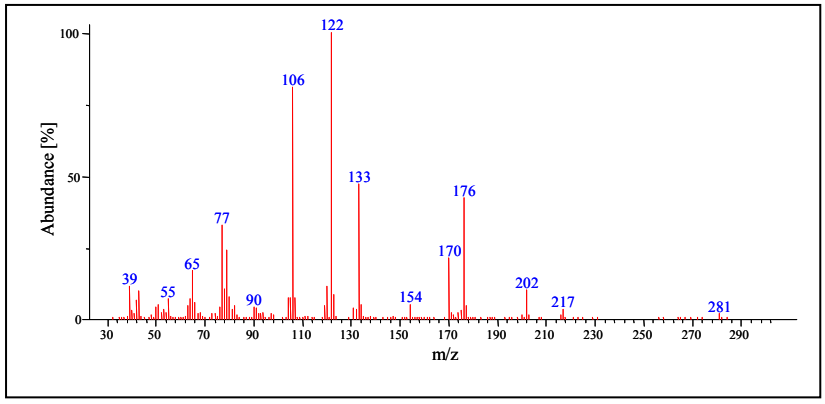


Fig. 3.69: Electron ionization mass spectra of the diazomethane derivative of an unknown Ac-SFM-artifact (?) formed under batch condition in surface water mix

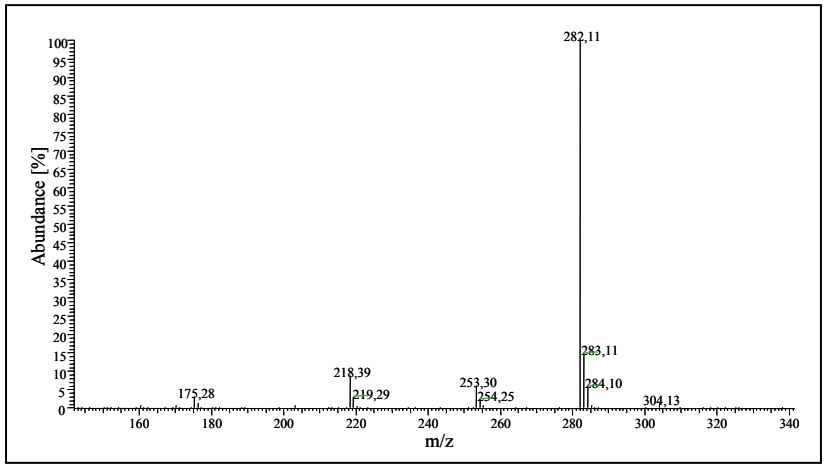


Fig. 3.70: LC-ESI$^+$/MS mass spectra of an unknown methylated Ac-SFM-artifact (?) formed during batch tests

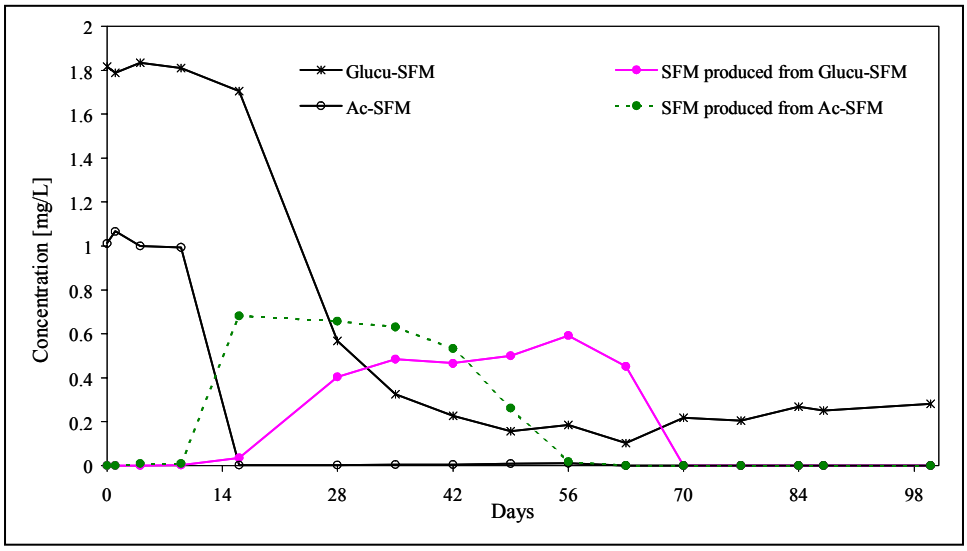


Fig. 3.71: The transformation of Glucu-SFM, Ac-SFM and the formation of SFM under batch condition in surface water mix (LC/UV)

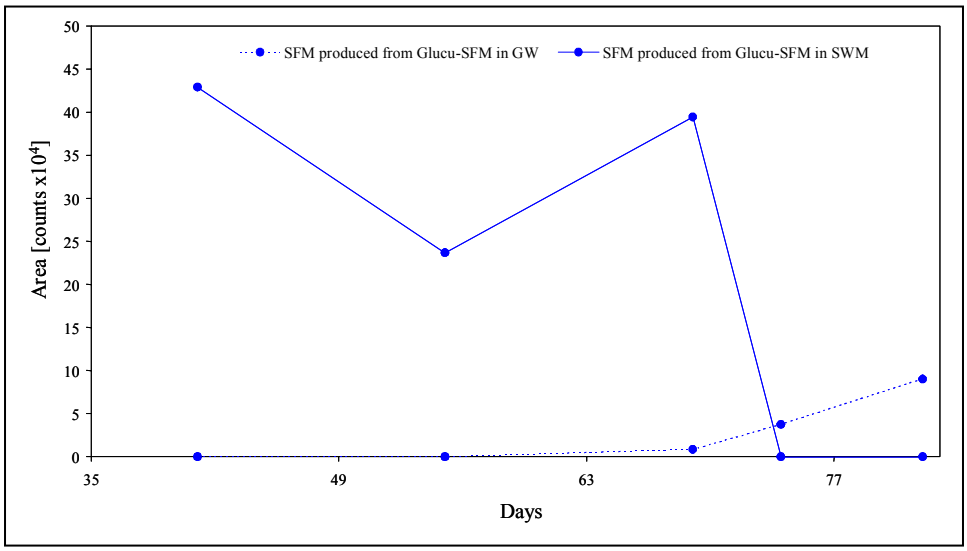


Fig. 3.72: The formation of SFM from Glucu-SFM metabolite under batch conditions (GC/MS)

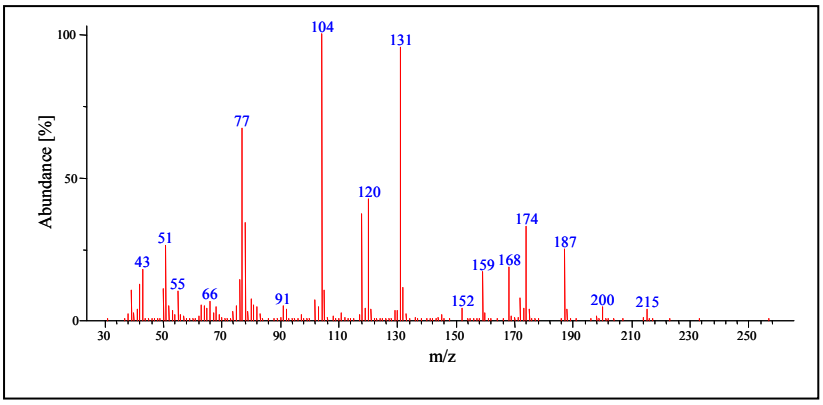


Fig. 3.73: Electron ionization mass spectra of the diazomethane derivative of an unknown Glucu-SFM-artifact (?) formed under batch conditions

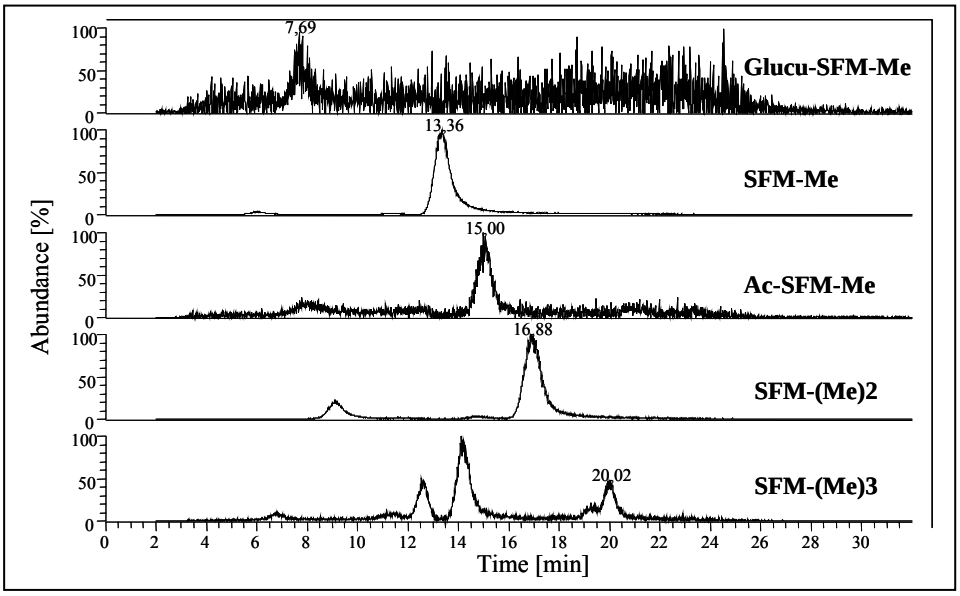


Fig. 3.74: Extracted mass chromatograms of an LC/MS run, as a result of a derivatization of a Glucu-SFM sample in surface water mix batch test

3.4.5. Conclusion

In this chapter, the biodegradation was carried out in long-term batch and biofilm reactor experiments for the target drugs; CBZ, DCF, IBU and SFM. The batches were run in present of culture medium under ground water (GW) and surface water affected by the run off of a wastewater sewage plant (surface water mix; SWM) from the river Ruhr, while the biofilm reactors were run under ground water and non loaded surface water (SW) from the same river. Moreover, some of their synthesised metabolites; Diol-CBZ, Ac-SFM, Glucu-SFM and OH-IBU were investigated under unsterile batch conditions in GW and SWM. The batch experiments were carried out both in sterile and unsterile for the selected drugs.

In order to discriminate between the products from the biodegradation tests and the present contaminants in the applied analytes, the purity of the commercial standards and of the synthesised metabolites were studied first by GC/MS and LC-ESI/MS.

The sterile batches revealed no degradation processes for all analytes during the tests time due to the absence of the bioactivity in the water matrix. Whereas, IBU and SFM were degraded significantly under unsterile SWM batch condition within 4-14 weeks depending on the bioactivity in the water matrix. In contrast to that, DCF and CBZ are not readily biodegradable under the applied operation conditions. Moreover, the synthesised metabolites are degraded at the same condition significantly in different rates within 2-9 weeks except Diol-CBZ, which was stable under the applied conditions.

Concerning the GW batches, SFM showed degradation as similar as in SWM but IBU showed long-term stability. It decreased after 6 months dramatically. Under similar conditions, DCF and CBZ and the synthesised metabolites showed no degradation.

In the biofilm reactors with GW and SW no degradation of the analytes was observed within 4 weeks. On the other hand, when cycling the test solution in surface water within a closed circuit column, a significant degradation within 7-10 weeks for all the analytes except CBZ could be observed.

In order to understand more about the degradation mechanisms, further verification based on LC- and GC/MS was adapted.

OH-IBU was identified as the major metabolites of IBU biodegradation in the unsterile ground water batch and surface water biofilm test. The other conditions showed degradation processes to unknown fates but no IBU metabolites could be identified.
In term of SFM biodegradation, low yield from Ac-SFM in unsterile GW, unsterile SWM and closed circuit column was observed as transformation product. Eventually, degradation leads to unknown products. CBZ and DCF didn't show any transformation products in all batches, whereas DCF showed the formation of an unknown-artifact in closed circuit column as detected by GC/MS.

OH-IBU as synthesised metabolites significantly degraded to unknown products in both batches. Whereas, Ac-SFM showed the formation of SFM in SWM batch. Additionally, in SWM batch an unknown signal was detected by GC/MS. Ultimately, the whole components degraded to unknown fate. Glucu-SFM degraded in a similar way as Ac-SFM. In GW and SWM batches SFM was detected as a transformed product from Glucu-SFM, in addition to two unknown products were detected by GC/MS.

4. Summary and conclusions

In this work the attention was turned to investigate the possibility of an application of particular animal intestines as natural membranes for the depletion of target drugs in water treatment and for sample preparation prior to specific laboratory analysis. Also, analytical methods for the simultaneous qualitative and quantitative determination of the selected analytes and further identification of unknown products should be developed based on liquid and gas chromatography and mass spectrometry. Moreover, long-term biodegradation of the analytes at particular conditions in batches and biofilm reactors should be investigated to estimate their fate in the aquatic environments.
The target drugs were carbamazepine (CBZ), diclofenac (DCF), ibuprofen (IBU) and sulfamethoxazole (SFM), furthermore, 10,11-dihydro-10,11-dihydroxycarbamazepine (Diol-CBZ), N-1-glucuronidesulfamethoxazole (Glucu-SFM), N-4-acetylsulfamethoxazole (Ac-SFM) and 2-hydroxyibuprofen (OH-IBU) as some of their main metabolites. The analytes were chosen as leading drugs for the present work according to the amount consumed in medicine, high concentrations occurrence in the aquatic environment and the varieties of physical and chemical characters.

Within the ***first part*** of the presented work it could be shown that membranes of natural intestines behave like dialysis membranes. A very favourable intestine is the so-called Goldschlägerhäutchen, a special part of the cattle appendix, which has optimal permeation characteristics and besides that, it is commercially available in large sizes in different formats and with low costs. Permeation processes by means of Goldschlägerhäutchen are mainly influenced by the concentration gradient, surface area and stirring velocity. Water ingredients such as surfactants can interfere the permeation process but only at unusual high concentrations. Humic substances interfere the permeation process of some analytes when its in young genesis. The depletion of the drugs can reach > 90 % by combination of the permeate solution with additional solid or liquid phase extraction.

When comparing the animal intestines to technical membranes, no clogging effects could be observed during the permeation through the natural membrane, even if the water sample contained a complex mixture of other water ingredients.

The stability of the natural membranes was not sufficient for all water matrices depending on the bioactivity. Particularly wastewater promoted instability for this membrane type. So, they might only be applied to technical application in combination with disinfection or filtration steps. A modifying treatment of the intestine with formaldehyde could extend the stability up to 10 days. But the best way would be to reduce the bioactivity of water.

Due to slow permeation kinetics the natural membranes cannot be recommended for analytical purpose, such as clean-up procedures.

In the ***second part***, GC/MS and LC-ESI/MS quantification methods were developed based on a pre-concentration step by solid phase extraction and analytical derivatization prior to the GC/MS analysis.

For sample preparation many attempts were done to find out the most proper adsorbent and derivatization agent. Oasis HLB as polymeric sorbent showed superior extraction recoveries for the target drugs and diazomethane proved to be the best reagent for derivatization.

A direct comparison of GC/MS and LC-ESI/MS displayed that the latter may have an advantage for the analysis of the extreme polar analytes due to incomplete derivatization of polar functional groups. Whereas, the LC-ESI/MS method is especially matrix dependent caused by high amounts of organic and inorganic ions in the sample, which lead to signal suppression.

In term of linearity, reproducibility and accuracy, both methods are applicable to the intended aims.

Applying the final procedures, the recoveries of the spiked analytes at constant sample volume (1 L) and concentration (1 µg/L) were 79-111 % for ultrapure water samples by means of GC/MS and 60-85 % using LC-ESI/MS. For surface water the recoveries were 91-129 % based on GC/MS and with LC-ESI/MS 46-78 %. The method detection limits were 1-5 ng/L for GC/MS (except SFM and its metabolites) and 3-5 ng/L in LC-ESI/MS for all analytes.

The developed methods were applied to real aqueous samples from the river Ruhr. All the target analytes could be detected in the surface water in a concentration range from 100 to 320 ng/L, except Glucu-SFM.

Thirdly, long-term biodegradation experiments in batch and in biofilm reactors were carried out in pilot scales at particular conditions for the selected drugs and some of their main metabolites.

The drugs in different batches showed different behaviour depending on the type of matrices and bioactivity as well. IBU and SFM were degradated significantly but in different rates. CBZ and DCF were resistant to the degradation processes under the applied conditions. The main metabolites were also degradated in a similar way to the parent drugs.

Except for CBZ, the biofilm reactor experiments significantly revealed a decline in the concentration of all target drugs at different rates.

Further samples were collected successively in order to follow the degradation of the target drugs and possible formation of metabolites. Different batches revealed the formation of OH-IBU as a major metabolite from IBU. Also Ac-SFM was identified as a metabolite from SFM under the applied conditions. Whereas, the human main metabolites did not show any remarkable degradation products under the batch conditions except the formation of SFM resulting from Ac-SFM and Glucu-SFM as well.

As a consequence, in long-term batch experiments IBU and SFM could be degradated under certain conditions. However, IBU, SFM and DCF were significantly degradated under specific biofilm reactor conditions. In contrast, CBZ was resistant to all applied conditions. Additionally, in batch experiments the degradation of the main metabolites showed similar behaviour compared to parent drug.

The batch and biofilm reactors have been proved to be suitable model systems especially for ground and surface waters in order to investigate the pathways of the biological degradation of active drugs as well as their main metabolites under particular conditions.

5. Experimental

5.1. Chemicals and materials

All chemicals and solvents were of purity high grades and used without further purification as described in (Table 5.1). Ultrapure water was prepared with a Seral-Pur Delta UV apparatus (USF Seral, Ransbach, Germany).

Different types of animal intestines were applied in the original wet form or dried as membrane.

- Pig : (small intestine; A91M, mast intestine with internal fat layer)
- Sheep : (small intestine; Nova ESS Kal 55/30, Nova ESS Sheets 50x50)
- Cattle : (special appendix part; Goldschlägerhäutchen)

The preferred membranes were special parts of the cattle appendix distributed under the trade name 'Goldschlägerhäutchen' (Jürging GmbH, Versmold, Germany). The intestine was stored under sodium chloride and was washed before use.

5.2. Sample preparation

The stock solutions of the selected drugs (25 mg/L) were prepared in ultrapure water.
Dissolution of the analytes was always done in the ultrasonic bath at 40 ºC for half an hour.

a) Sample clean up and extraction

The suspended particles in the aqueous samples were vacuum filtered through cellulose membrane filter (0.45µm).

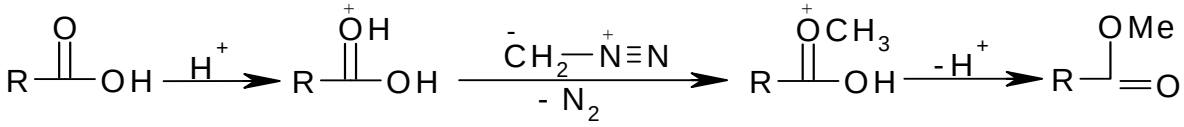
A 100 mg Oasis HLB cartridge was conditioned first with 6 mL MeOH, 6 mL MeOH-EtOAC (50:50 v/v), and 3 mL MeOH, followed by 12 mL acidic water (pH ≈ 2). The filtered water samples were sucked through the cartridge with a flow-rate of 5 mL/min. followed by washing with 3mL fresh water. Then the cartridges were dried with vacuum under a gentle stream of nitrogen. The analytes were eluted from the cartridge first by 3 mL MeOH under ground gravity, and followed by 3mL MeOH: EtOAC (50:50 v/v). The elutes were collected in a 10 mL test tube and concentrated to about 300 µL by using a heating block at 40 °C under a gentle stream of nitrogen. Then the sample was divided in 2 halves in order to analyse the same sample with GC and LC methods.

For LC/MS analysis the sample was dried and the residue redissolved to a final volume of 200 µL with buffer A (10 mM Ammonium acetate (pH 4)) containing 10 % MeOH. For GC analysis the sample was dried and the following derivatization step was carried out in an adequate organic solvent.

b) Derivatization procedures applied

1) Diazomethane (CH_2N_2):

Diazomethane is a yellow gas but is used in the form of an ethereal solution. Its reacts with an organic acid in the following manner,



1 mL diazomethane solution was added to the analytes in a 1.5 mL vial. It was capped tightly and then the reaction was allowed to occur within 60 minutes in the refrigerator. Later on the solution was evaporated under a light stream of nitrogen at room temperature. The residue was dissolved with EtOAC for GC/MS analysis.

The hint for security must be followed because diazomethane is carcinogenic and can be extremely unstable with a risk of explosion. All reactions should be carried out in a fume hood and any stored solutions of diazomethane in diethyl ether should be restricted to a maximum volume of 100 mL and kept in a refrigerator [84].

Diazomethane preparation: [85]

4.2 g Diazald (N–methyl-N–nitrous-p-toluene sulfonamide) was dissolved in 50 mL diethyl ether in a separator funnel. 10 g NaOH was dissolved in 40 mL H_2O and 30 mL MeOH in a round-bottom flask. The special distillation apparatus with clear seals was connected tightly in a fume hood. The round bottom flask was warmed up to 40-45 °C by the heating mantle. The diazald solution was dropped from the separators funnel into the sodium hydroxide solution very slowly. The released diazomethane gas was collected in the ice bath as a diethyl ether fraction.

2) Trimethylsulfonium hydroxide (TMSH): [86]

The analytes were redissolved with EtOAC and then 5 µL TMSH was added; the solution became a little bit turbid, therefore, 1 µL (10 % acetic acid in EtOAC) was added to consume the excess of TMSH. It's suitable to measure it directly with GC/MS.

3) Pentafluorobenzyl bromide (PFBB): [87]

The analytes were redissolved with 1 mL acetone, then they were treated with 10 µL PFBB, 30 µL 0.1 mg/mL dicyclohexyl-18-crown-6) and 10 mg powdered potassium carbonate (K_2CO_3). The vial was capped tightly and the reaction was allowed to occur within 30 minutes at 60 °C. After that the solution was isolated and dried under nitrogen. The residue was dissolved with EtOAC for GC/MS analysis.

4) Silylation reagent (I): [88]

Mixture I: 95 % N,O-bis(trimethylsilyl)acetamide (BSA) and 5 % trimethyl-chlorosilane (TMCS)

The residue was redissolved with 100 µL from mixture I and then the reaction solution was heated for 2 h (120 °C). Later on it was dried under nitrogen and the rest was redissolved with EtOAC for GC/MS analysis.

5) Silylation reagent (II): [89]

Mixture II: N-methyl-N- (trimethylsilyl) trifluoroacetamide (MSTFA)/trimethyl-silylimidazol (TMSI)/dithioerytrite (DTE), 1000 µL/2µL/2mg (30 min at 80 °C).

The residue was redissolved with 50 µL of mixture II and then it was heated for 30 min in a water bath (60 °C). Later on it was dried under nitrogen and the rest was redissolved with n-hexane for GC/MS analysis.

5.3. Instrumentation parameters

a) UV-VIS-NIR

The absorbance was measured by using a Cary 5 G UV-VIS-NIR spectrophotometer (Varian, Darmstadt) at varied wavelengths, for the HUS a wavelength of 254 nm was applied.

b) LC-UV

Method I

An analytical method based on HPLC (Sykam, Gilching) and UV-detection (Spectra-Physics, Darmstadt) has been developed.

Wavelength	: 225 nm
Analytical column	: Nucleosil-120 C-18 (5 µm, 125 x 4 mm i.d., Knauer, Berlin)
Pre-column	: Nucleosil-120 C-18 (5 µm, 10 x 4 mm i.d., Knauer, Berlin)
Mobile phase	: Acetonitrile/NaH_2PO_4-buffer (55/45 v/v)
Flow rate	: 0.8 mL/min (isocratic elution)
Injection volume	: 5 or 20 µL

Method II

Using the same conditions (Method I) with the exception of the mobile phase

Mobile Phase	:10 mM NH_4CH_3COO: ACN 75:25 (v/v) (pH: 6.0).

Method III

The analytical method used to analyse the PEG tests was based on using HPLC (Merck, Germany) and RI detection (Water, USA).

Stationary phase	: (300 x 16 mm i.d.) column was packed with Fractogel TSK HW-40S (particle size 20-40 µm, pore size 50 Å and separation range PEG: 100-3000; Grom GmbH)
Mobile phase	: Ultrapure water
Flow rate	: 1 mL/min

c) LC-ESI/MS

The LC/MS system was a LCQ Deca (Thermo, USA) coupled with a spectrasystem gradient pump (P4000; Thermo) and a spectrasystem autosampler (AS 3000; Thermo).

Analytical column	: Aquasil C-18 (5 µm, 100 Å, 150 x 0.32 mm i.d., ThermoHypersil)
Mobile phases	: *Mobile A*: 10 mM Ammonium acetate (pH = 4.13) adjusted with concentrated acetic acid *Mobile B*: 10 mM Ammonium acetate in 98 % (ACN: MeOH 40:60)
Gradient	: Eluent B: 25% to 60% for 10 min, increased to 90 % within 4 min, held for 5 min at 90 %, then ramped back to 25 % in 5 min and held finally for 6 min at 25 %
Flow rate	: 13 µL/min; Split 200 µL/min, the mobile phases were degassed with an online spectrasystem degasser (SCM 1000) using helium
Injection volume	: 10 or 20 µL
Spray voltage	: 3.5 kV (+ mode) and 3.2 kV (- mode)
Capillary temperature	: 200 °C
Sheath gas flow rate	: 0.3 L/min N_2
Sheath liquid	: 4 µl/min (isopropanol/water (5:1))

d) GC/MS

Separation and detection of the analyte was carried out with the GC/MS system HP G1800A GCD with autosampler 7673A (Hewlett Packard, Ratingen).

Capillary column	: A fused-silica (HP-5MS; 30 m; 0.25 mm ID; 0.25 µm FD)
Injector	: Split/splitless, splitless time of 2 minutes
Injector temperature	: 250 ˚C
njection volume	: 1 µL
Carrier gas	: Helium
Flow rate	: 1 mL/min
Temperature program	: Oven = 60 °C (1.5 min), first ramp: 20 ˚C/min to 120 ˚C, second ramp 4˚C/min to 160 ˚C, third ramp 12 ˚C/min to 250 ˚C and then hold 12 min at 250 ˚C
Analysis time	: 34 min
Interface temperature	: 280 ˚C
Scan mode	: Full scan range (m/z = 30-450)
Ionization mode	: EI^+/ 70 eV

5.4. The conditions of the batches and biofilm reactors

The ***batch tests*** followed the guidelines of the 'Organization for Economic Cooperation and Development' (OECD) published in 1993 [108]. The guidelines represent a standardized method, specially the MITI-Test, to test the biodegradation. The principle of this test is the determination of the biodegradation potential of chemicals under favourable conditions. Simply, the pharmaceuticals provide as carbon source. They are dissolved in culture medium and are addited to the water matrix containing the natural bioactivity.

The test was carried out with the model substances CBZ, DCF, IBU and SFM in ground water and from the river Ruhr surface water affected by the run off of a wastewater sewage plant. The experiments were prepared parallel in sterile and unsterile water.

Culture medium:
65.25 mg K_2HPO_4, 25.5 mg KH_2PO_4, 133.8 mg $Na_2HPO_4.2H_2O$, 5.1 mg NH_4Cl, 67.5 mg $MgSO_4$.7H2O, 82.5 mg $CaCl_2$ and 0.75 mg $FeCl_3.6H_2O$; the final volume: 1 L.

Batch solution:
1 mg/L of the 4 analytes each were dissolved in the culture medium containing 16 % real water.

The ***column experiments*** were performed at the pilot plant station by the Institute for Water research in Schwerte-Geisecke. The columns set up are shown in (Fig. 3.59).

The technical data for the biofilm reactor were as follows:

Column height	: 100 cm
Column diameter	: 22 cm
Filter height	: 80 cm
Flow rate	: 1.6 L/h
Backing material	: Rhine sand (0.2-2 mm particle size; River Rhine, Germany)

The columns were filled with Rhine sand as supported material on which the biofilm was grown by feeding the columns with ground or surface waters.

The content of the microorganisms in surface water was characterised by a comparison with ground water as the following:

1 mL water sample is given on a plate with culture medium DEV (Merck Nr. 1.11471.5000). One plate of each probe is incubated at a temperature of 20+/-2°C and one plate at 36+/-1°C. After 44+/-4 h incubation time the colony number of each plate is counted (visible colonies at a 6fold to 8fold loupe amplification).

After the hydraulic, chemical and biological conditions had been stabilized the real waters were spiked with 100 µg/L of the analytes each and sucked through the columns.

Table 5.1: Chemicals and materials used in the present work

Chemical	Supplier
Acetic acid	Aldrich
Ammonium acetate	Aldrich
Ammonium chloride	Merck
Ammonium hydroxide	Merck
ß-Cyclodextrine	Fluka
Calcium chloride	Merck
Diazald	Aldrich
Dicyclohexyl-18-crown-6	Merck
Ethylenediaminetetraacetic acid	Merck
Ferric chloride- 6-hydrate	Merck
Hydrochloric acid	Baker
Magnesiumsulfate-7-hydrate	Merck
Polyethylene glycol	Fluka
Polysorbate (TWEEN 80)	Sigma
Potassium carbonate	Merck
Potassiumdihydrogenphosphate	Merck
Potassiumhydrogenphosphate	Merck
Sodium dodecylsulfate	Merck
Sodium hydroxide	Fluka
Sodium sulfate	Fluka
Sodiumdihydrogenphosphate	Merck
Sodiumhydrogenphosphate-2-hydrate	Merck
Sulfuric acid	Merck
Tris(2-ethylhexyl)-phosphate	Merck
Solvent	
Acetone Suprasolv	Merck
Acetonitrile Rotisolv HPLC	Roth
Decan puriss.	Fluka
Dichloromethane Suprasolv	Merck
Diethylether Seccosolv	Merck
Ethyl acetate Pestilyse	Roth
Formaldehyde purum	Merck
Methanol Pestilyse	Roth
n-Decanol purum	Fluka
n-Hexane Suprasolv	Merck
Octanol purum	Merck
Reference Compound	
Carbamazepine	Ehrenstorfer
Diclofenac	Ehrenstorfer
Ibuprofen	Ehrenstorfer
Sulfamethoxazole	Ehrenstorfer

Reference Compound	Supplier
3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169)	Ehrenstorfer
N-4-Acetyl-sulfamethoxazole	Synthesised at University of Paderborn
10,11-dihydro-10,11-dihyroxycarbamazepine	Synthesised at University of Paderborn
2-Hydroxyibuprofen	Synthesised at University of Paderborn
N-1-Glucuronide sulfamethoxazole	Synthesised at University of Paderborn
Hohlohsee 13	Natural material from Hohloh Lake (Germany)
Venner Moor	Natural material from Arnsberger Wald (Germany)
Reagent	
Dithioerytrite	Fluka
N,O-bis(trimethylsilyl)acetamide	Fluka
N-methyl-N- (trimethylsilyl) trifluoroacetamide	Fluka
Pentafluorobenzyl bromide	Aldrich
Trimethylchlorosilane	Merck
Trimethylsilylimidazol	Analyt
Trimethylsulfonium hydroxide	Machery&Nagel
Material	
Cellulose ester dialysis membrane	Reichelt Chemietechnik GmbH
Goldschlägerhäutchen	Jürging GmbH
Membrane filter (0.45µm)	Schleicher-Schüll
SPE Material	
Charcoal	Merck
Lichrolute EN	Merck
Oasis HLB	Waters
Octadecasilane (C-18)	Restek GmbH
Equipment	
Dialyse Chamber	Self-production
Heating and drying block	Techne DRI-Block DB-20
Micro magnetic stirrers	H+P Labortechnik AG

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7. Publications and presentations of the present work

I) Publications

1. M. Grote, B. Haciosmanoglu, **M. Bataineh**, J. Nolte, Separation of drug traces from water with particular membrane systems, J. Environ. Sci. Health Part A, Vol. A39, No. 4, pp. 1035 - 1049, 2004.

2. J. Nolte, **M. Bataineh**, B. Haciosmanoglu, M. Grote, Membrane systems developed for the separation of pharmaceutical residues from water, in: M. Cox: Ion Exchange Technology for Today and Tomorrow, SCI-Verlag, London, 2004, pp. 259-266, ISBN 0-901001-85-6.

3. B. Haciosmanoglu, M. Grote, J. Nolte, **M. Bataineh**, Separation of drug traces from contaminated water with particular membrane systems, Proceedings of the 'ISWA World Environment Congress & Exhibition' Istanbul, 8 - 12.7.2002 Eds.: G. Kocasoy, T. Atabarut, I. Uholgu; In: Appropriate Environmental and Solid Waste Management and Technologies for Developing Countries 3, 2002, pp. 1801-1808, ISBN 975-518-179-2.

II) Oral presentations

1. **M. Bataineh**, M. Grote, B. Haciosmanoglu, J. Nolte, Extraction of pharmaceuticals from water by use of natural flat- und liquid-membrane systems, Trends in sample preparation, 29.6 - 4.7.2002, Graz, Austria.

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3. **M. Bataineh**, J. Nolte, B. Haciosmanoglu, M. Grote, Membrane systems developed for the separation of pharmaceutical residues from water, IEX 2004, 4 - 7.7.2004, Cambridge, UK.

III) Poster presentations

1. J. Nolte, **M. Bataineh**, U. Marggraf, H. Geltenpoth, M. Grote, Development of a method for the enrichment of pharmaceuticals from waters using selected natural membranes, Euroanalysis-12, 8 - 13.9.2002, Dortmund, Germany.

2. **M. Bataineh**, U. Marggraf, H. Geltenpoth, M. Grote, J. Nolte, Nachweis von Diclofenax - Oligromeren mittels ESI-MS, Jahrestreffen der Deutschen Gesellschaft für Massenspektrometrie (DGMS 2003), 10 - 12.03.2003, Münster, Germany.

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