Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt
Department für Biowissenschaften
Lehrstuhl für Ökologische Chemie und Umweltanalytik
Technische Universität München

Development and Application of Analytical Methods for the Determination of Methimazole and its Metabolites and their Effects to Thyroid Homeostasis in Zebrafish (*D. rerio*)

LASZLO HOLLOSI

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für
Ernährung, Landnutzung und Umwelt der Technischen Universität München
zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof.Dr. rer. nat. habil. Wilfried Huber

Prüfer der Dissertation:

1. Univ.-Prof. Dr. rer. nat. Dr. h. c. (RO) Antonius Kettrup
2. Univ.-Prof. Dr. rer. nat. Dr. agr. habil.
   Dr. h.c. (Zonguldak Univ. / Türkei) Harun Parlar
3. Priv.-Doz. Dr. rer. nat. Dr. agr. habil Karl-Werner Schramm

Die Dissertation wurde am 23.03.2005 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 08.06.2005 angenommen.

ACKNOWLEDGEMENT

I would like to acknowledge GSF Research Centre for Environmental and Health for the financial support that allowed me to undertake this Ph.D. thesis.

I would like to offer my thanks to my “Doktorvater” Prof. Antounius Kettrup for his confidence and pleasant guidance and support.

I would like to thank my project supervisors Dr. Karl-Werner Schramm and Dr. Rupert Simon for their guidance and the many useful instructions during my work and for giving me a vote of confidence.

My thanks have to go to Dr. Gerd Pfister for the supporting me during the hormone analysis and to Dr. Philip Schmitt-Kopplin for the opportunity to use capillary electrophoretic systems and the very friendly climate.

Many thanks to Dr. Istvan Gebefügi and Dr. Jenö Fekete for their support and good advices.

Many thanks also to my colleagues and friends Kris, Antje, Andreas, Naima, Marchella, Stephan, Geza, Reinhard, Frau Danzer, Ernst for the nice memories and the unforgettable moments. Also many thanks to Annette for her precise and accurate work.

And last but not least all my love and thank to my mummy and daddy, and also to Agi for being there, supporting me and always having faith in me.

ABBREVIATIONS

μ_{eff}: effective mobility

$\mu_{eff,norm}$: normalised effective mobility

ACN: acetonitrile

ADS: alkyl-diol silica

APCI: atmospheric pressure chemical ionisation

BCF: bioconcentration factor

BGE: background electrolyte

cAMP: cyclic-adenosilmonophosphate

capLC: capillary liquid chromatography

CE: capillary electrophoresis

CEC: capillary electrochromatography

CNS: central nervous system

CZE: capillary zone electrophoresis

DAD: diode-array detection

DIT: dioiodo-thyronine

ED: electrochemical detection

ESI: electrospray ionisation

EOF: electroosmotic flow

EPA (US-EPA): Environmental Pollution Agency

FIA: flow injection analysis

FMO: flavin-monooxygenase

GC: gas chromatography

GPC: gel permeation chromatography

GCB: graphitic carbon blacks

HILIC: hydrophilic interaction chromatography

HLB: hydrophilic-lipophylic balance

HPLC: high performance liquid chromatography

ICP-MS: inductively coupled plasma with mass spectroscopy

I.D.: inner diameter

IEX: ionic exchange

IUPAC: The International Union of Pure and Applied Chemistry

k´: capacity factor (retention factor)

LC: liquid chromatography
LC-MS: liquid chromatography-mass spectrometry
LLE: liquid-liquid extraction
LOD: limit of detection
LOQ: limit of quantification
M: molecule mass
MAX: mean anionic exchanger
MEKC: micellar electrokinetic chromatography
MeOH: methanol
MS: mass spectrometry
MEH: N-methylhydantoin
MET: methimazole
MIT: monoiodothyrosine
MSPD: matrix solid phase dispersion
MSO: N-methylimidazole-2-sulfinic acid and N-methylimidazole-2-sulfonic acid
MSOH: N-methylimidazole-2-sulfinic acid
MSO2H: N-methylimidazole-2-sulfonic acid
MT: migration time
MTU: N-methylthiourea
N: theoretical plate number
OEHHA: Office of Environmental Health Hazard Assessment
PCBs: polychlorinated biphenyls
PGC: porous graphitic carbon
ppb: parts per billion
ppm: parts per million
Q: charge
Q-TOF: quadropole-time of flight mass analyser
R^2: regression coefficient
RIA: radioimmunoassay
RP: reversed phase
R_S: resolution factor
RSD: relative standard deviation
rT3: reverse triiodo-thyronine
SEC: size exclusion chromatography

SHMET: 2-mercaptoimidazole

SIM: selective ion monitoring

SPE: solid phase extraction

T3: 3,5,3´-triiodo-thyronine

T4: thyroxine

TEA: triethanolamine

TH: thyroid hormone

TLC: thin layer chromatography

TOF: time of flight mass analyser

TSH: thyroid stimulating hormone

t_r: retention time

TRH: thyreotropine releasing hormone

UV: ultra violet

VB: breakthrough volume

VIS: visible

CONTENT

1. INTRODUCTION AND GOALS

The last half-century has shown a dramatic increase in the standard of living millions of people all over the world. This has, in many ways been brought about by scientific and technical developments which were indicated initiated in the 1940s and 1950s. Promises were made that scientific and technical attainments would revolutionise our manufacturing industry and our way of life. Whilst some of these promises were fulfilled, the problems of (long-term) health risks to humans and wildlife arising from the use, production and disposal of these products were either unknown or deliberately understated. One of the final resting points of all these chemicals is the aquatic ecosystem. Residues of e.g. organochlorine pesticides and other chemicals remain in sediments and are still being taken up into fish tissues decades after their production was discontinued. During the last decades there has been increasing evidence that many of the chemicals are not only toxic at high concentrations, but can cause disruption of the endocrine system at levels which do not cause obvious ill-health. Numerous other chemicals which underpin the present lifestyle of the western society may be having similar effects.

Both humans and wildlife, whether they live in cities or in the wilderness, are now exposed to bewildering array of chemicals in the food they eat, the air they breathe and the water they drink. The obsession of the western world with cancer has to a great extent obscured the evidence that many chemicals may, at very low levels, cause endocrine disruption both in humans and animals. Only during the last few years has such evidence finally attracted the attention of the public authorities and funding been given to research. Endocrine disruption is now a rapidly expanding field of research, and has shown that some of the chemicals which have for long been claimed to be inert are in fact capable of causing widespread disruption of the endocrine systems of both laboratory animals and wildlife. Few of the chemicals to which we and or wildlife are exposed have been tested for such activity and toxicity testing now needs to be redesigned to include the risk of possible endocrine disrupting activity in both humans and the wildlife.

Localized studies have found evidence of reproductive abnormalities in fish exposed to discharges from sewage treatment plants, paper mills, and other industrial or chemical pollution sources. Among these abnormalities are fish with depressed levels of naturally produced hormones (like estrogens and androgens), fish that exhibit both male and female characteristics (intersex fish) in normally separate sex species, degraded gonadal structure, and male fish that have elevated levels of a protein usually associated with egg laying in females. The various endocrine-disrupting compounds affect fish differently - some mimicking and others counteracting the action of naturally occurring hormones to create an assortment of reproductive abnormalities. Although effects on reproduction are typically the focus of endocrine disrupter studies, the authors noted that other important functions influenced by the endocrine system, such as metabolism or growth, could also be targets of endocrine disrupters, although more research is needed in these areas.

Current safety testing of chemicals concentrates on lethal or carcinogenic effects which have little relevance to the potential long-term low level effects which characterise endocrine disruption, yet alone transgenerational effects which may not become apparent until the second or even the third generation attains adulthood. Recent workshops have shown that the design of such tests requires a detailed knowledge of normal endocrine function and some clear indication of which endocrine parameters might be best used as the endpoints of such tests.

Fish, which are at the greatest risk from pollution of the aquatic environment, a suitable laboratory test animal, and an early-warning of potential short- and long-term health effects in humans. Although fishes differ from mammals in the detail of their endocrine systems, the underlying basis is essentially the same, so that chemicals which affect fish have a very high probability of having similar harmful effects in human. Studies of endocrine disruption in mammals rely overwhelmingly on experimentation with laboratory animals since mammalian wildlife is not readily amenable to examination of low-level effects and they are available only in limited numbers. Fishes are good study objects furthermore because they are easy to work with, their susceptibility to endocrine disruptors has been validated by laboratory experiments.

These aims were expressed even in the final recommendation of the US-EPA Risk Assessment Forum 1997: future efforts should concentrate on developing improved tests to identify environmental agents that alter endocrine function through their action. Such tests are needed to identify any adverse neuroendocrine changes that occur in response to exposure during development and/or in adulthood.

Clearly, there is a need for protocols and multiple tests to identify chemicals that have the potential of disrupting thyroid hormone function. In rat studies, propylthiouracil treatment during development impairs CNS function (i.e., hearing) in adulthood.

Information on effects of chemicals in both sexes and the effects of exposure to the fetus, children, and adults are necessary. Once these apical tests are developed and validated, then additional tests to ascertain mechanisms of action seem appropriate. In an effort to extrapolate test animal to human equivalence, reasonable dose-response data are needed, along with pharmacokinetics studies.

Compared with the studies on the reproductive function, relatively little is known about the effects of pollutants on the thyroid tissues at molecular biological levels.
Considering to these claims, our research activity aimed investigations of the endocrine disruption of the thyroid axis at molecular and molecular biological levels. Effects of environmental pollutants on the thyroid hormone relevant genes and their expressions in zebrafish (*Danio rerio*) were set to study. Thus, there is a huge number of chemicals (pesticides, PCBs, heavy metals) with certified thyroid disrupting activity, we choose methimazole, a known anti-thyroid agent as test substance to mimic thyroid disruption effects.

GOALS

To ascertain that changes in thyroid function are aftermaths of the presence of the test substance, chemical analytical method had to develop to verify if test substance really penetrated into the tissues. Taking in to the consideration, that not just the mother substance, but even its metabolites can be affected on various function of an organism, we aimed the *development of an analytical method for the simultaneously determination of methimazole and its metabolites* from the zebrafish tissues and from the experimental environment. Another claim of the project was to *develop* further *analytical method* for monitoring the changes of *the thyroid hormone levels* in the fish tissues to verify the following disruption of the thyroid axis after administration of the disrupter substance.

High performance liquid chromatographic and capillary electrophoresis methods were chosen for the determination of the endocrine disruptor and its metabolites, considering to their performance and versatile applicability. Since the determination of the thyroid hormones desired more sensitivity and efficiency, capillary liquid chromatographic technique with mass spectrometric detection was applied for the analysis. Developing of adequate sample preparation methods were also desired to increase the sensitivity and the selectivity of the analytical method and to decrease the effect of the very complex matrix on the analytical process.

Determination of the changes at the molecular biological level was carried out in the scope of a supplementary doctoral thesis, and the results were compared.

2. THEORETICAL BACKGROUNDS

2.1. ENDOCRINE DISRUPTION

The endocrine system is the chemical communicational system of the body which regulates such activities as body fluid homeostasis, management of stress, and reproduction and fertility which are necessary for propagation of the species. It comprises glands such as the testes, ovaries, pituitary and hypothalamus, thyroid and liver. It has become increasingly apparent in the scientific literature over the last two decades that the endocrine system is particularly sensitive to very low levels of pollutants. Any anthropogenic chemical which disrupts normal activity of the endocrine glands and the production of the hormones which they secrete, can cause profound disruption to the exposed animal.

The definition of an endocrine disruptor is not perhaps as clear as may be first thought. Clearly a compound that mimics a natural hormone, like e.g. environmental estrogens, can be considered an endocrine disruptor. Equally well, a pollutant that affects synthesis or metabolism of a hormone, its binding to cellular receptors or its expression in gene transcription can be considered as a disruptor.

The frontiers of endocrinology have expanded greatly and are no longer so clearly defined a sin the past. The original definition of a hormone was a compound which was released from an endocrine gland into the blood by which it was transported to its target tissue where it exerted its effect. It is now clear that some hormones act within the gland in which they are produced, and may even act on neighbouring cells or within the same cell without passing into the circulatory system. This applies especially to some of the gonadal hormones, but the field is now wide enough to encompass a range of intracellular messengers. Endocrine disruption can therefore apply, not only to the classical range of endocrine function, but also to this extended area of intracellular communication. A wide range of developmental processes are under hormonal control, as is obvious in pubertal development, but it is often not clear whether abnormal development is a result of a direct toxic effect on cellular differentiation and division, or the result of disruption of the subtle effects of hormone action. Many of these developmental changes are certainly encompassed by the more liberal interpretation of endocrine action which covers intracellular communication. This is especially important during embryonic development, which is not only affected by classical hormones, such as steroids and thyroid hormones of embryonic or maternal origin, but also intracellular messengers which determine the development of the embryo. Disruption of embryonic development resulting in mortality or malformation of the fry may, therefore, also be considered to be the result of endocrine disruption interpreted in its broadest sense. Reflecting the increase in scientific, popular and political concern about environmental endocrine disruptors over the last few years, a number of workshops have been held to discuss the actions necessary to assess the safety of anthropogenic chemicals and to define more clearly an endocrine disruptor.

Kavlok et al. broadly defined an endocrine disruptor as an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes. Another, perhaps simpler definition put forward at the Weybridge workshop in 1996 (European Commission, 1996) defined: an endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or in its progeny, consequent to changes in endocrine function, while " a potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism.

FISH AS BIOMONITORS OF ENVIRONMENTAL POLLUTION

Fishes possess a similar physiological system to mammals, including humans, and many of the pollutant induced disturbances in fish are likely to have similar effects in humans. In particular, the reproductive endocrine system has a great deal of similarity within the vertebrates and effects found in fish may be readily applicable to other vertebrate systems. If bioaccumulation of pollutants in fish causes ill effects, that in fish-eating communities may be expected to extreme severe.

As Fishes, even human societies are exposed to a wide range of chemicals, in their water, air, household goods and foods. Pollutants remain in vegetables, meat and fish, and processed foods contain high levels of chemical additives. Many of these have not been rigorously tested for long-term effects-fish provide both a relevant model for such tests and warning of general environmental deterioration resulting from the general release of these chemicals.

Our aquatic ecosystems have increasingly been the depository for pollutants for over half a century. Many of these pollutants remain in the sediments from which they will be absorbed into the aquatic organisms and hence up the food chain to large carnivorous fish and marine mammals.

Compared with the studies on the reproductive function, relatively little is known about the effects of pollutants on the functions of the thyroid tissues. This undoubtedly due to the fact that in fish, unlike mammals, this tissue does not form distinct gland, but is scattered in small clumps which makes experimental isolation and examination more difficult. There is considerable overlap in function between the thyroid and interrenal axes with regard to control of growth and metabolism. It is therefore appropriate to consider these together with related endocrine functions such as the stress response, which also involves the chromaffin tissue analogue of the mammalian adrenal medulla, osmoregulation and factors affecting growth.

The thyroid gland in fish, as in mammals, is primarily concerned with regulating metabolic rate which may in turn affect both activity and growth of the fish. Growth may be, for example, be stimulated by thyroid treatment, although the results are closely dependent upon the conditions under which fish are maintained [*Barrington et al.,1961*]. Conversely, growth is inhibited by irradiation which decreases or abolishes activity of the thyroid gland [*Norris, 1969; Brown, 1997*]. The thyroid is also involved in a less specific manner with larval development, behaviour and reproduction and there are interactions between the growth hormone, the gonadal hormones and thyroid function [*Matty,* 1985]. There is also evidence that the thyroid may be involved in the parrsmolt transformation, the spawning migration and osmoregulation [*E.G. Grau*, 1987].

Recently, bony fish species are favourised for endocrine disruption studies. The embryos undergo rapid cleavage, and the embryonic development is essentially completed within 24 to 72 hours [*Liu and Chan,* 1997]. Zebrafish (*D. rerio*) completes the embryogenesis and hatches in about 3 days. In addition, they are not only easy to work with and maintain, but also the genetic staff is widely studied [*Vittozzi and De Angelis*, 1991].

2.2. THE THYROID GLAND

Unlike mammals in which the thyroid is a discrete gland situated around the trachea, that of teleosts forms diffuse follicles scattered around the ventral aorta and its branches. Fishes possess a similar physiological system to mammals, including humans, and many of the pollutant induced disturbances in fish are likely to have similar effects in humans. Therefore we summarise the function of the thyroid system in fish by to the presentation of the human knowledge.

The main function of the thyroid gland is to manufacture and release the thyroid hormones thyroxine (T4) and triiodothyronine (T3). These two hormones maintain metabolic stability by regulating intermediate metabolism, body weight and oxygen requirements. They are also important in the normal growth and development of the body.

The hormones are produced from the precursor amino acids mono and diiodotyrosine which are synthesised following iodination and coupling of the thyrosine residues on the protein thyroglobulin synthesised by the follicular cells (Figure 1.). The coupling reaction appears to occur separately from iodination and is catalysed by thyroid peroxidase. Thyroglobulin is a large molecule with a molecular weight of 660.000 and is present as a soluble protein in the lumen of the thyroid follicle.

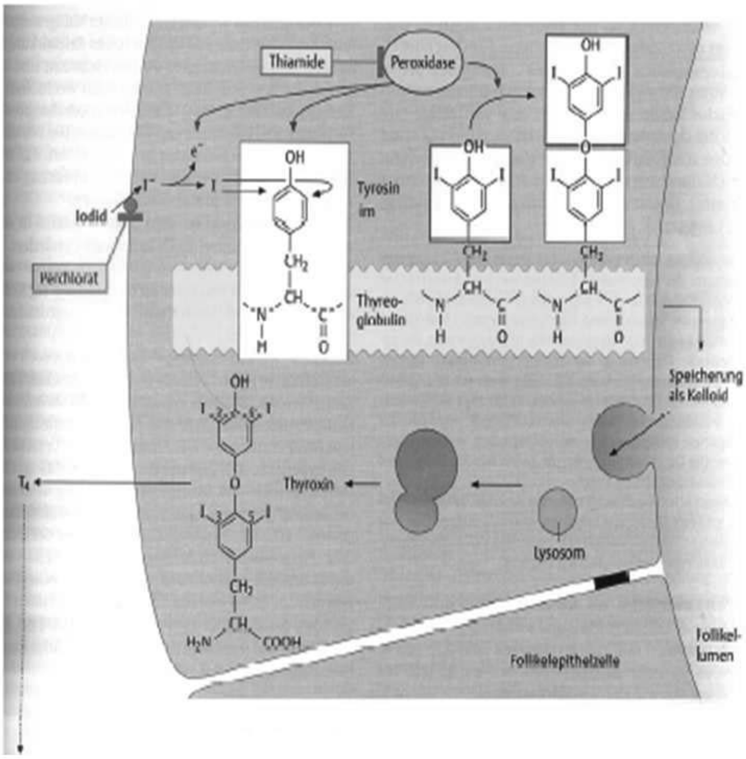


Figure 1. Schematic description of the thyroid hormone synthesis in the thyroid follicles

In contrast to most endocrine glands, the thyroid gland is able to store 2-3 months supply of thyroid hormones in the thyroglobulin pool. The thyroglobulin must be hydrolysed to release T3 and T4. The cleavage is accomplished by lysosomal proteases within the cells of the follicle. The process starts with the action of thyroid stimulating hormone (TSH) which stimulates the endocytosis of luminal colloid and the fusing of lysosomal hydrolytic enzymes.

The freed thyroid hormones (THs) diffuse from the cell into circulation where approximately more than 90% are bound to carrier proteins (Table 1).

Table 1. Properties of the main human thyroid hormone carrier proteins

Property	TBG	TTR	SA
Serum concentration [mg/l plasma]	15	300	45000
Binding capacity [µg/l]	200	2300	100
Affinity for T4 at the strongest site [M^{-1}]	10^{10}	10^{8}	10^{6}
%T4 carried	75	15	10
%T3 carried	50	25	25
Physical properties			
Molecular weight	54000	54000	66000
Peptide chain number	1	4	1
β-structure %	40	45	15
α-helix %	30	1	48
Chemical properties			
Half-cystines	2	4	35
S-S bridges	0	0	17
Total carbohydrate	23	0	0

Some binding of thyroxine to serum lipoproteins is also observed in a number of species (*Benvenga et al*., 1994) and, in an isolated case, to vitamin D-binding protein. An important biological advantage of such a network systems is its increased capacity for adaptation: if one component becomes deficient, the remaining parts of the system can compensate for this deficiency.

The binding of hormones to the carrier proteins is not because of their insufficient solubility in aqueous phase. The dependence of the solubility of thyroxine in aqueous media on the pH is illustrated in the table 2. The solubility of the T4 at pH7 was found to be 2.3 μM, while the concentration of the total (free and protein bound) T4 in plasma is about 100 nM, that is clearly below the solubility limit. Therefore, in contrast to a frequently found textbook error, insufficient solubility of T4 in plasma does not necessitate the presence of carrier proteins; the bondage serves rather as buffer.

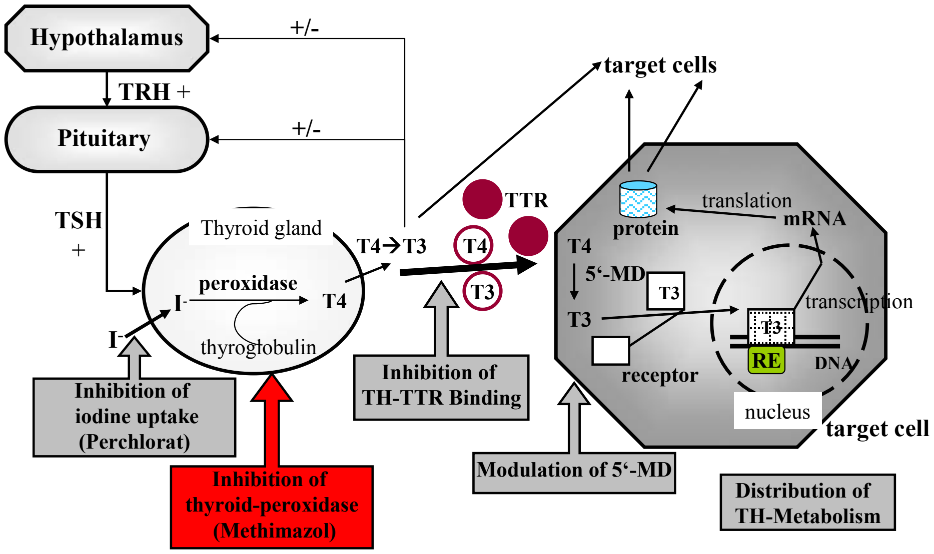
Table 2. Water solubility of the thyroxine depending on the solvent pH

	Solubility of thyroxine	
pH	mg/l	μmol/l
1	1.6	2.1
2	1.6	2.1
3	1.2	1.4
4	1.4	1.8
5	1.4	1.8
6	1.6	2.1
7	1.8	2.3
8	2.0	2.6
9	3.4	4.5
10	21.0	27.0
11	202.0	260.0
12	1631.8	2100.0

No literature data was found about the carrier proteins in zebrafish. As Schreiber et al. reviewed, opposite to mammals, only albumin take part in the fishes in the TH bounding and carrying. However zebrafish was not investigated, we suppose that this is also valid for the *D. rerio*.

The thyroid gland is mainly regulated by TSH from the anterior pituitary (figure 2.). TSH acts on a membrane bound receptor on follicular cells to increase the concentration of the intracellular second messenger, cAMP. The release of the TSH by the pituitary is under the control of the tripeptide hormone TRH via the median eminence - pituitary portal blood system and the negative feedback influence of the thyroid hormones upon thyrotrophs. TRH stimulates thyrotrophic exocytosis by cleaving Ca2+ levels using an IP3 mechanism. The release of TSH is not wholly dependent upon TRH since there is some background release. TRH is released by neurons that project towards the median eminence and release their hormone in a pulsatile fashion. It is probable, but not proven, that the thyroid hormones also act to inhibit (possible negative feedback) on TRH-producing neurones in the hypothalamus. TSH release, in addition to its tonic secretory control by TRH, is also under the inhibitory control of somatostatin (GHIH) and dopamine.

The majority of biological actions of thyroid hormones appear to be mediated by an interaction of T3 with nuclear receptors and the resultant stimulation of transcription of specific mRNA`s. THs stimulate the number of Na+/K+ATPase pump units and thus increases oxygen consumption in those tissues that have nuclear thyroid hormone receptors. Protein synthesis and degradation are enhanced by thyroid hormones as are most aspects of carbohydrate metabolism. In this respect THs may have a permissive action in that they allow the actions of adrenaline and insulin to be maximised. The TH effects on lipid metabolism is complex and is thought to involve the expression of low-density lipoprotein receptors on the cell membrane surface.



I^- – iodine**; RE** – responsive element; **TH** – thyroid hormone; **TTR** – transthyretin;
TRH – thyreotropine-releasing hormone; **TSH** – thyreotropine; **T4** – 3,3`,5,5`-Tetraiodo-L-thyronine;
T3 – 3,5,3`-Triiodo-L-thyronine; **5`-MD** – 5-monodeiodinase

Figure 2. Schematic overview of the thyroid axis regulation. Arrows represent the possible inhibition sites.

The serum half life of T4 is about 7 days whereas the half life of T3 is less than a day. Plasma T4 level is higher than T3 , and T4 is converted to T3 in most tissues suggesting that T4 is a prohormone. This deiodination step is supposed by 5-deiodinase, which and 5´-iodinase are responsible also for the degradation of the thyroid hormones (Figure 3).

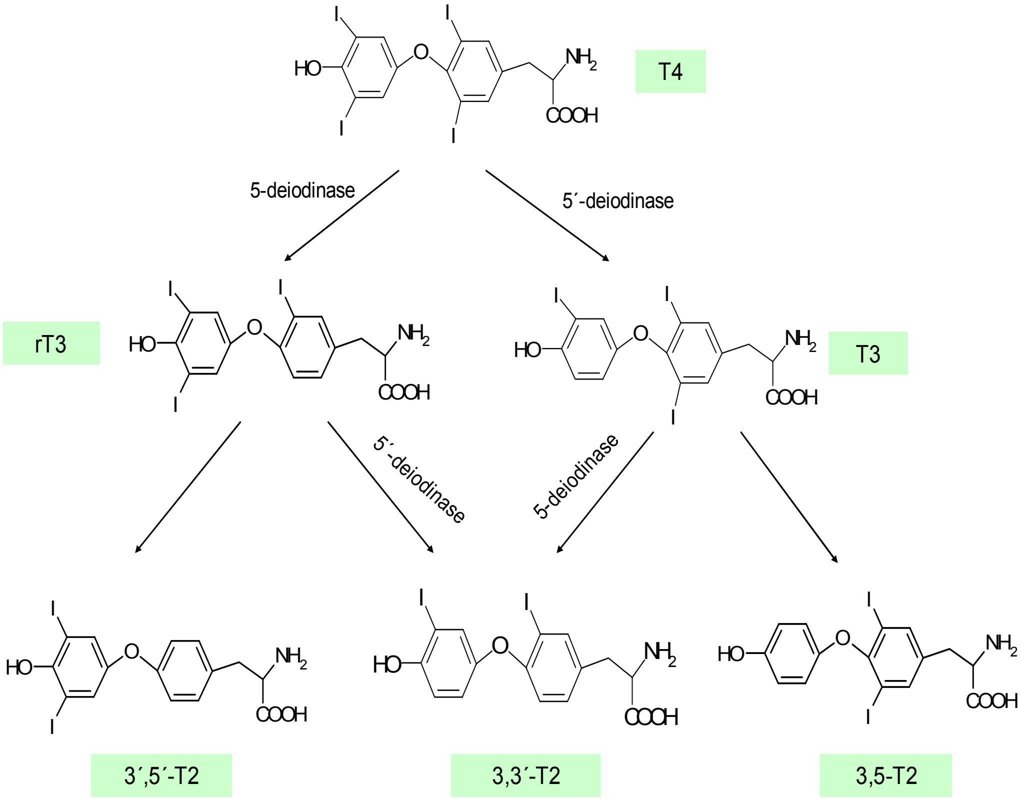


Figure 3. Metabolism of the thyroxine (T4). Also T3 is formed in the enzymatic process

According to the specifications of clinical chemistry laboratories (Klinkum Grosshadern in Munich and Heinrich-Heine Universitätsklinik, Duesseldorf), the human reference values vary between ~2-5 ng/l for free T3 and 9-19 ng/ for free T4, respectively. Total T4 concentration was measured in the range between 51-135 µg/l and 0.8-2 µg/l for T3.

According to Kwon et al., thyroxine concentrations in rockfish (*Sebastes inermis*) tissues were found as 16.4-119.3 µg/l. Carletta et al. determined 4-45 ng/g T4 and 0.8-2.8 ng/g T3 in

Fundulus heteroclitus. Any information about the TH concentration in zebrafish was not found in the literature.

2.3. ANALYTICAL METHODS FOR MEASUREMENT OF THE THYROID HORMONES

Competitive protein assays, [*Bigos et al.,* 1988; *Murphy et al.*, 1964] flow injection analysis by inhibition of glutamate dehydrogenase, [*Ghous et al.* 2000] magnetic particle-based solid phase fluorogenic electrochemiluminescent immunoassay [*Yu*, 1998] and electrochemiluminescent immunoassay (*M. Sanchez-Carbayo et al.* 1999) methods were applied for the determination of the thyroid hormones, but radioimmunoassay (RIA) [*Nuutila et al.,* 1978] become the most widely used analytical technique for the screening of the thyroid hormones in the last decades. Even clinical chemical laboratories apply this kind of method due its simply usable and high sensitivity. More advantageous is, that the method can be easily automated (with high measurements per hour ratio) and the method is available in commercial kit form. But the relative high costs, the possible cross reactions and unspecificity [*Soukhova et al.* 2004] to even totally other structures, the complicated and limited assay development potential, and the limited versatility of the method arouse the need of a more versatile analytical technique which unites the advantages of the immunoassays [*Vogeser* 2003]. Chromatographic methods are alternative to that, considering that the separation methods are usable for the simultaneously analysis of different types of substances and provides both qualitative and quantitative information.

However, the sensitivity of the separation techniques with commercial detection methods are fare above the sensitivity of the immunoassay techniques, this can be reduced with modern detection systems and selective and specific sample pre-treatment.

Chemical derivatisation followed by gas chromatography with electron capture detection [*Petersen et al.* 1976], high performance liquid chromatography with electrochemical detection [*Takahashi et al.* 2002] were established for the higher sensitivity, but spectacular increasing of the efficiency was obtained with using mass spectrometric detection. Michalke et al. developed a powerful hyphenation of HPLC and ICP-MS [*Michalke et al.* 2000]. Sodium-thyroxine and its degradation product were determined from Na-thyroxine tablets by HPLC with APCI-Q MS detection [*Kazemifard et al.* 2001]. Isotope dilution mass spectrometry techniques [Tai et al. 2002; *De Brabandere et al.* 1998; *Siekmann*, 1987; *Gika et al.* 2005] were taken as basis for our method development. Soukhova et al. published a direct comparison of an immunoassay and an isotope dilution mass spectrometric method [Soukhova et al. 2004].

Table 3 gives a short overview of the reported liquid chromatographic methods for the determination of thyroid hormones considering the matrices and especially sample preparation technique.

Table 3: Literature overview about the most important thyroid hormone determination methods by separation techniques (ID: isotope dilution mass spectrometry, n.d.: no data).

Sample (matrix)	Sample preparation	Method	LOD	Ref.
Pharmaceutical preparation	Dilution, fluorescence derivatisation	HPLC-FD	0.25 ppm	Takahashi, 2002
Tablets	Dilution	HPLC-APCI Q MS/ECD	n.d.	Kazemifard, 2001
Human serum	Acidic hydrolysis, LLE, SPE	GC-ESI MS (ID: $^{13}C_2$-T4)	n.d.	Siekmann, 1987
Human serum	Acidic hydrolysis, LLE	HPLC-ESI-Q MS (ID:$^{13}C_6$-T4)	1.2 ppb	Brabandere, 1998
Human serum	Acidic hydrolysis, LLE, SPE	HPLC-ESI-Q (ID: T4-D_5)	1 ppb	Tai, 2002
Animal serum	LLE	cGC-MS (ID: $^{13}C_9$-T4)	50 ppb	Hantson, 2004
Pharmaceuticals, human blood and urine	SPE	HPLC-DAD	0.1 ppm	Gika, 2004
Human serum	Dilute and shot	HPLC-ESI-Q (ID: T4-D_2)	0.062 ppb	Soukhova, 2004

2.4. METHIMAZOLE AS ENDOCRINE DISRUPTOR

Methimazole (Figure 4.), 1-methyl-2-mercaptoimidazole, is a widely used in medicine for the treatment of hyperthyroidism, and as adjuncts in the treatment of thyrotoxicosis or thyroid storm. [*Velkeniers et al.* 1998; *Cooper*, 1986; *Tapazole package insert* ; *Laurberg et al.* 1986; *Bouma et al.* 1982; *Romaldini et al*. 1983; *Laurberg et al.* 1985; *Miller,* 1979]

In the last years methimazole became also a fashioned model substance for endocrine disruption (thyroid axis) studies thus inhibits the production of the thyroid hormones. Further application of methimazole as model substance in biochemical studies based on its alternative FMO and cytochrome P-450 substrate function [*Mizutani et al.* 1999; *Krause et al.* 2002]

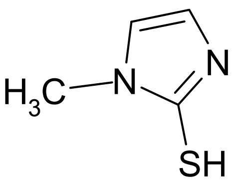


Figure 4. Chemical structure of methimazole (1-methyl-2-mercaptoimidazole)

The most important pharmacokinetic properties of the methimazole obtained in human are summarised in the followings:

Methimazole inhibits synthesis of thyroid hormone within the thyroid gland by serving as substrates for thyroid peroxidase, which catalyzes the incorporation of oxidized iodide into tyrosine residues in thyroglobulin molecules and couples iodotyrosines. [*McDougall et al.*1981; *Cooper*,1984]. Methimazole diverts iodine from the synthesis of thyroid hormones [*Jansson et al.* 1985; *Okamura K et al.* 1987; *Cooper*, 1984]. The antithyroid agent do not interfere with the actions of exogenous thyroid hormone or inhibit the release of thyroid hormone [*de los Santos et al.* 1990], therefore, stores of thyroid hormones must be depleted before clinical effects will be apparent [*Gilman* 1990]. Antithyroid agent may also have moderating effects on the underlying immunologic abnormalities in hyperthyroidism due to Graves' disease (toxic diffuse goiter), but evidence on this point reported to date is inconclusive

Oral bioavailability of the methimazole is 93%, according to human investigations [*Sitar*, 1990], but absorption may be unpredictably affected by food [*Jansson* et al.]. Methimazole is actively concentrated by the thyroid [*Okamura et al* 1987; *Okune et al.,* 1987*;* *Jansson R, et al.* 1983], the volume of distribution is approximately 0.6 liter per kilogram (L/kg) of body weight [*Mammen,* 1989].

Methimazole binding to proteins is not significant [Langhorne, 1989; Okamura et al. 1987]**,** the half-life is about 5 to 6 hours [*Jansson et al.,* 1985; *Kampmann et al.* 1981; *Nabil et al.* 1982]. In one study, substantial reductions in mean serum thyroxine and triiodothyronine concentrations were seen after 5 days of methimazole therapy at 40 mg per day [*Roti, et al.,* 1989]. Peak serum concentrations were measured as 1.184 ± 0.12 µg/mL (blood concentrations), after administration of a 60-mg dose to healthy subjects, while 1.163 ± 0.15 mcg/mL blood concentration was measured after rectal administration of a 60-mg suppository to healthy subjects. Less than 10% of the adsorbed methimazole is excreted in the urine unchanged, [*Kampmann et al*. 1981] the total body clearance is approximately 10 L per hour. In a two-year study, thyroid hyperplasia, adenoma, and carcinoma have developed in rats when given methimazole at doses of 3 and 18 mg per kg of body weight per day (mg/kg/day) (2 and 12 times the 15 mg/day maximum human maintenance dose calculated on the basis of surface area) [*Product Information: Tapazole*®]

METHIMAZOLE IN THE ENVIRONMENT

Since methimazole was widely used in the human medicine in the last decades, also the environmental pollution by methimazole became an interesting aspect taking its effect on aquatic living beings into consideration. No literature data were found about the environmental load originated from methimazole, but the California Environmental Protection Agency’s Office of Environmental Health Hazard Assessment (OEHHA) as lead agency for the implementation of the Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65) has developed a procedure for prioritizing chemicals for consideration under Proposition 65 by the “State’s qualified experts”, and announced in 2003 a draft data summaries for 50 chemicals selected for prioritization with respect to their potential to cause cancer, and Methimazole was categorized within the top 10 candidates due to high carcinogenicity concern. The procedure used by OEHHA to identify, prioritize and select candidate chemicals is available on the internet at www.oehha.ca.gov .

2.4.1. METHIMAZOLE METABOLITES

Methimazole is metabolised primarily hepatic [*Skellern et al.,*1980]. According to the literature data, methimazole is metabolised rapidly. As studies with rat tissues ascertained, methimazole disappeared completely from the incubation mixtures [*Taurog & Dorris*, 1989]. As 35S-labelled measurements had shown, the earliest detectable 35S-labelled product was the methimazole-disulphide, which reached a peak after a few minutes and then declined to undetectable levels. Coincident with the decrease in disulfide was the appearance of two 35S peaks, the major corresponding to sulfate and/or sulfite, and the other to an unidentified component “with 7.5 min. elution time”. This 7.5 minutes metabolite was not found in rat samples in vivo.

The thyroidal accumulation followed by intrathyroidal oxidisation of methimazole to sulphate was observed in Phenobarbital treated rats. [*Lees e*t al. 1997].

Toxicological studies with mice demonstrated, that methimazole causes liver injury characterised by centrilobular necrosis of hepatocytes and an increased serum alanin-transaminase activity. Methimazole requires metabolic activation by both P450 monooxygenase and flavin-containing monooxygenase (FMO) before it produces the hepatotoxicity. The hyphotesis was verified, that methimazole undergo epoxidation of the C-4,5 double bond by P450 enzymes and, after being hydrolysed, the resulting epoxide would be then decomposed to form N-methylthiourea, a proximate toxicant [*Mizutani* et al., 2000]. N-methylthiourea was also identified as urinary metabolite of methimazole in mice.

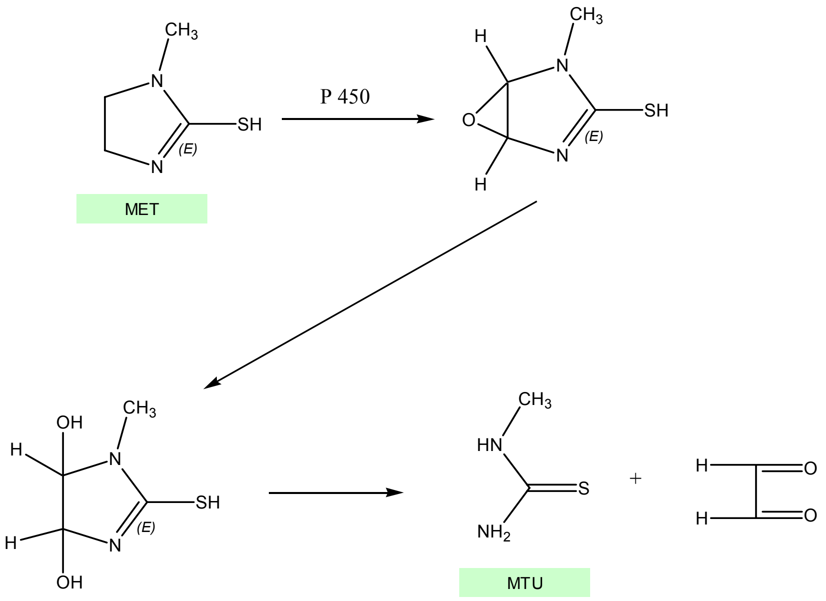


Figure 5. Formation of the hepatotoxic and teratogene N-methylthiourea due to the metabolism of the methimazole. Methimazole is oxidized by cytochrome P450 oxidase in the liver.

No literature data was found about metabolism of methimazole in fish. Therefore, we decided to search for all in other species identified metabolites (MSO, MTU) and in addition for not yet identified, but eventually possible metabolites, which represent further important metabolising step of a vertebrate.

As (Mizutani et al.) presented, epoxidation of the C4-5 must be occurred prior to forming MTU as end-product. Since epoxide can be easily transformed to oxo forms, we also tested N-methylhydantoin as other oxidised metabolite and 2-mercaptoimidazole as metabolite forming by demethylation. A summary of the metabolites of methimazole for our investigations are seen in the figure 6.

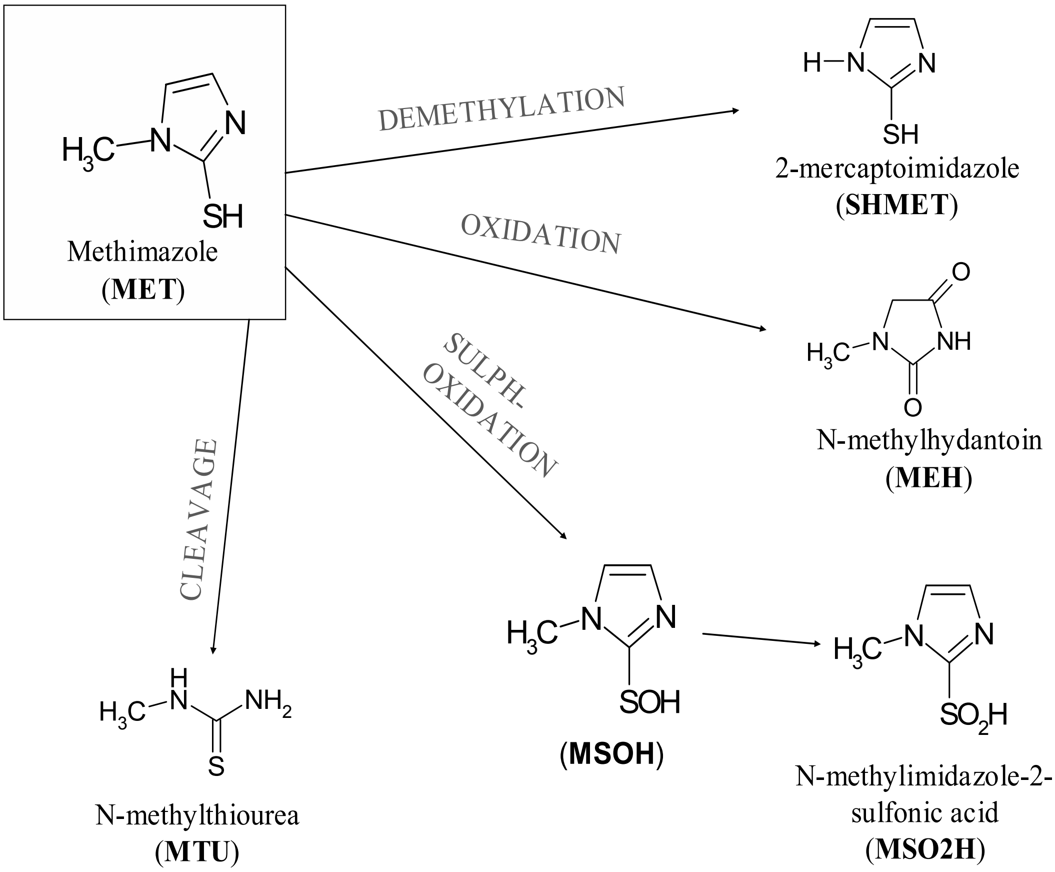


Figure 6. Structures of the investigated methimazole metabolites with the art of their formation

2.4.2. ANALYTICAL METHODS FOR MEASURING METHIMAZOLE AND METABOLITES

Various techniques have already been reported for analysis residues or methimazole in meat [*Hooijernik & de Ruig,* 1987], urine [*P Batjoens* et al. 1996; *Ienaga et al.* 1988; *Lawrence et al.* 1981], blood [*Tatsuhara et al.* 1985; Kobayashi et al. 1981; Kobayashi et al. 1982; *Moretti et al.* 1993;) vegetable samples [*Kontou et al.* 2001]. Most of these methods are capable for severally identification of methimazole, ethylenethiourea, prophylthiouracil and other thiouracil derivates. Techniques were used like thin layer chromatography [*Aletrari et al.* 1998], high performance liquid chromatography with UV [P*ochard et al.* 1984] and/or electrochemical detection, flow injection analysis [*Sanchez-Pedreno et al.* 1995] gas chromatography with nitrogen-phophorus or mass spectrometric detection [*Buhlert,* 1986;

Schilt et al. 1989; *Moree-Testa et al.*, 1984]. Methimazole was determined with other thiols by capillary zone electrophoresis with amperometric detection [*Whang et al.* 2000].

These methods were able to determine these compounds separately but no method is known to be able to determine methimazole and its metabolites simultaneously from biological samples. The selection of the most important methods in the literature is listed in the table 4 below.

Table 4. Short summary of the known analytical methods for the determination of methimazole and its metabolites

Reference	**Component**	**Matrix**	**Method**	**Sample preparation**
Tatsuhara, 1985	MET	serum	HPLC-ED	Deproteinisation and dilution
Moretti, 1993	MET, thiouracile derivates	serum	HPLC-UV	LLE
Aletrari, 1998	MET	Pharmaceutical product	TLC	dilution
Sanchez-Pedreno, 1995	MET	Tablets	FIA	Dilution
Duarte, 2000	MET, prophylthoiuracil	plasma	HPLC-UV	LLE
Hashash, 2002	SHMET	Pharmaceutical product	HPLC-UV	Dilution
Batjoens, 1996	MET, thiouraciles	urine	GC-MS	LLE, derivatisation
Ienaga, 1988	MEH	Rabbit urine	HPLC-UV	dilution
Kobayashi, 1981	MTU, other thoiureas	plasma	HPLC-UV	LLE
Kobayashi, 1982	MTU	Rat plasma	HPLC-UV	LLE, SPE

2.5. ANALYTICAL METHODS

2.5.1. HIGH PERFORMANCE LIQUID CHROMATOGAPHY

IUPAC defines chromatography as: “chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase), while the other moves (mobile phase) in a definite direction. It may be liquid, gas or a supercritical fluid, while the stationary phase may be a solid, a gel, or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process”.

Two components, which are associated with the separation that occurs in a chromatographic system are the mobile and the stationary phases. In HPLC, the mobile phase is liquid delivered under high pressure to ensure a constant flow rate, and thus reproducible chromatography, while the stationary phase is packed into a column. Chromatographic separation occurs if the components of a mixture interact to different extents with the mobile and/or the stationary phases and therefore take different times to move from the position of sample introduction to the position at which they are detected. The chromatographic performance can be described in terms of a number of theoretical parameters (Table 5) although the performance required for a particular analysis will depend upon the separation which is required. This, in turn, depends upon the similarity in the behaviour in the chromatographic system of the analytes of interest to each other and to other compounds present in the mixture

Table 5. Summary of the most important chromatographic terms and their definition

Parameter	Sign	Definition
Dead time	t_0	$t_0=L/U$
Retention time	t_R	$t_R=t_M+t'_R$
Capacity factor	k'	$k'=(t_R-t_0)/t_0$
Selectivity factor	α	$\alpha=t_{R2}/t_{R1}=k'_2/k'_1$
Theoretical plate height	H	$H=16\left(\frac{t_R}{w_{0.1}}\right)^2$
Resolution	Rs	$Rs=\frac{1}{4}\sqrt{N}\frac{\alpha-1}{\alpha}\frac{k}{k+1}$

HPLC modes of separation may be broadly classified as gel-permeation (GPC), normal phase, partition, ion-exchange and reversed phase chromatography.

Gel permeation (or size exclusion) chromatography separate compounds based on their molecular size and it is generally used for the separation of biological macromolecules. In normal phase (adsorption) chromatography substances are separated on the polar stationary phase with non-polar mobile phase. The compounds are eluted in order of increasing polarity and the retention decreases with increasing solvent polarity. Compounds, which are highly soluble in organic solvents, such as lipids, fat soluble vitamins are usually by adsorption chromatography. However highly polar and hydrophilic compounds which are difficult to retain on reversed phase columns may also be separated on normal phase columns with polar eluents.

The partition chromatography based on the partition of the solute between the mobile and stationary phase. Chemically bonded normal phases, in which polar groups (CN, NH_2, NO_2 Diol) are chemically bonded to silica, may be considered as alternatives to the classical partition chromatography.

Ion exchange chromatography separates compounds based on their relative molecular charge. Separation is achieved because ions of opposite charge to that on the stationary phase surface are retained to different extents. Clear, that this technique is only applicable for ionisable compound. The retention is governed by the ionic strength of the buffer, while the pH of the eluent influences the resolution.

Reversed phase chromatography is the reverse of the normal phase chromatography. The stationary phase contains non-polar and hydrophobic surface, while compounds will be eluted by polar mobile phase.

Instead of more detailed description of the different chromatographic techniques, we now give an overview about the separation possibilities of polar compounds, what the aim of this thesis was.

Possible choices for separation of polar substances are chromatographic techniques like normal phase chromatography or reversed phase ion exchange chromatography, thus polar interaction (hydrogen-bridge) or ionic interaction between surface silanol groups or ionic exchange groups of the column surface and the polarized or ionized parts of the analytes. The by the pharmaceutical industry enthusiastic favoured reversed phase chromatography due its characteristic is not really suitable for the retention and separation of the polar substances. Many developments occurred nowadays and occurred in the last years (decades) aimed at a better retention and separation of polar compounds even on the preferred reversed phase stationary phases*, so* (ion pair) reversed phase and hydrophobic interaction chromatography are another possible techniques for the retention of polar compounds. Short comparison of these techniques is listed in the table 6.

Table 6. Short comparison of the chromatographic techniques suitable for separation of polar substances

	Advantage	Disadvantage
Ion exchange chromatography	Retention ionisable compounds	elution with high buffer concentrations (MS incompatibility) • no retention for non-ionisable compounds
HILIC	Increased sensitivity (MS) due the increased amount of organic part in eluent	• solubility problems • high re-equilibration time
Ion pair RP HPLC	• retention of ionisable substances • polar and non-polar separation on the same column (run)	• high equilibration time • problematic gradient elution • MS incompatibility
RP HPLC	• high efficiency • rapid equilibration • many types of the phases available • common used	• dehydration and collapse of the phase in aqueous eluent • no satisfactory retention of polar substances

Ion exchange chromatography is suitable only for separation of positive or negative ionisable compounds. Similar to that is the ion pair chromatography, what is for separation of ionised and hydrophobic compounds on the same column and run.

HILIC is for retention of polar and basic substances, while reversed phase chromatography is unique solid phase whit retention potential of polar and non-polar compounds both. The question, how to separate polar compounds in reversed phase chromatography, is discussed in the following part.

2.5.2. RETENTION OF POLAR SUBSTANCES ON REVERSED PHASE COLUMNS

As it was outlined above, the retention capacity of polar or ionisable compounds according to chromatographic criteria is very low on the reversed phase columns, mostly these compounds are dead volume markers, so while not to be able to be determined qualitatively or quantitatively. An optimal reversed phase suited for retention of polar substances results a good retention with good peak symmetry (even basic compounds), narrow peak width and should be compatible with all detector types. Good retention of polar substances often requires high aqueous ratio (>90-95 % H2O) in the eluent, but in several cases even 100% water is needed as eluent. The conventional reversed phase materials, especially C_8- and C_{18}-phases, are not convenient to working with high aqueous medium, because of the possible phase collapse. The reason of this phenomenon is the surface of the solid phase. It depends on:

- the nature of bonded phase
- the density of the bonded phase
- pore diameter of the particles.

As mentioned, the reason for that is the solid phase and its´ dewetting with the mobile phase. If eluent does not wet well the surface and (more important) the pores, retention loss or non-reproductive retention times, tailing or unreliable solvent composition can appear. The collapse does not damage the column, washing with more than 50% organic solvent can regenerate the column. However, after a period of time using a low percent of organic solvent, phase collapse can recure. Phase collapse can be avoided by using columns especially designed for operation in highly aqueous environment. What kind of columns are they?

COLUMNS

Twenty years ago, the low-purity silica gel used for most HPLC columns caused high surface acidity and tailing problems with basic compounds. The first modified stationary phase were the so-called base-deactivated bonded silicas. These were not deactivated with base, but the term implied that the column had been treated to provide minimal interaction and tailing with strongly basic compounds. As treatment was acidic washing of the silica before bonding, single or multiple reactant endcapping, covering or encapsulating the surface with polymeric phase and other methods. Later came to the market the type B materials with less acidic surface, more homogenous distribution of silanol groups and more- reproducible and denser bonding of alkyl chains. Even with these improved alkylsilane surfaces, certain experimental conditions can cause separation or solid phase problems. For example both very high (pH>10) and low (pH<2) pH conditions can present problems with column stability. For very polar substances, other problems can arise: the above mentioned phase collaps.

Several aspects of the solid phase design can help retain polar analytes under highly aqueous conditions, including:

- non-endcapped, short chain alkyl phases
- hydrophilic, polar-endcapped or polar enhanced phases
- polar embedded alkyl phases
- long-chain alkyl phases
- wide pore diameter phases.

2.5.2.1. NON-ENDCAPPED, SHORT CHAIN ALKYL PHASES

When unreacted silanols on the surface are present, they impart a degree of polarity that sometimes can be useful in the retention. The presence of this silanols can cause polar functionality on analytes, while the alkyl chains carry the non-polar function.the resulting mixed mechanisms can yield improved separations, but the packings might be difficult to produce batch to batch unless the ligand-to-silanol ratio is exactly the same. Surface silanols also interact with water (hydrogen bonding).

2.5.2.2. HYDROPHILIC, POLAR ENDCAPPED AND POLAR ENHANCED STATIONARY PHASES

Using polar or hydrophilic endcapping along with bonding of longer alkyl chains such as C_{18} is a successful development approach for stationary phases that can retain polar analytes reproducibly under highly aqueous conditions. So, these polar or hydrophilic endcapped chemicals allow the silica surface to be wetted with water and allow the full interaction with the longer alkyl chains. The bonding and endcappinmg process for this type of reversed phase packing is minimally a two step process. In the first step, the monochloroalkylsilane (C_8 or C_{18}) is bonded to silica. The second step uses the special endcapping reagent. Polar or hydrophilic endcapping chemicals could be short-chain trimethoxy- or triethoxysilanes, which can be hydrolysed after bonding to produce silanol groups. These silanols near the surface would provide a high degrre of polar character to the final alkyl bonded phase, but they have a lower acidity than residual silanol, found on the surface of bounded silicas. Another approach is to modifying polar or hydrophilic surfaces involves the use of polymeric bonding reagents e.g. octadetyltrimetoxysilane. Another way to achieve a polar enhanced surface is to incorporate polar or polar-embedded groups in alkyl chains. Ether group is used effectively as polar enhancing agent [*Przybyciel & Santangelo* 1998]. The ether group can be incorporated in the alkyl chain near the silica attachment point. This modification is polar enough to allow water to enter the pores of the silica. Without endcapping, many of these alkyl ether phases can be used at low pH levels.

2.5.2.3. POLAR EMBEDDED ALKYL PHASES

Another successful method for applications with highly aqueous mobile phases is using polar-embedded alkyl stationary phases, sometimes called polar-linked phases. With the incorporation of a polar functional group in the alkyl ligand close to the surface of the silica gel, the phase remains solvated by water at low percentage of organic modifier and even with 100 % water. Under these conditions, the alkyl chains maintain their conformational freedom and can interact with polar analytes. It is also believed that the presence of the polar functionality close to the surface shields the effects of unreacted silanol groups. The polar embedded concept has many advantages:

- the stationary phase maintains a reversed phase character
- the phases provide different selectivity compared with alkyl phases, particularly with polar analytes
- the phases can be used in low percentage of organic solvent or with 100% water without dewetting
- silanol activity is suppressed, which leads to better peak shapes and decreased tailing of basic compounds, particularly at intermediate pH values.

2.5.2.4. LONG CHAIN STATIONARY PHASES

Increasing the hydrophobicity of the stationary phase can generate a sufficient amount of hydrophobic interaction between the stationary phase and cause the hydrophobic portions of the analytes to increase the retention of polar analytes. Generally, these phases are more retentive for polar non-polar analytes, than are most polar-embedded and even high-coverage C_{18} phases. Because of a higher degree of surface shielding, long-chain phases also offer greater pH stability than do C_8 and C_{18} phases. Surprisingly, the long-chain phases are also more resistant to phase collapse under high aqueous conditions than are C_{18} phases. This behaviour could be related to conformation changes in the stationary phase with temperature. The melting point of C18H38 which is the functional group of a typical C_{18} phase, is 29-30°C, however, the melting point of $C_{30}H_{62}$ is 68-70°C. Therefore, C_{30}, the triacontyl ligand, is in a solid state at 30-40°C, which is the typical operating temperature of a reversed phase column, and its ligand does not move or collapse under highly aqueous mobile phase conditions *[Nagae & Enami* 2002]. The C_{18} phase is in a liquid state and its ligand moves easily or collapses under highly aqueous mobile phase conditions. Interestingly, Nomura Chemical Co. recently received a US patent in which the claims cover the use of C_{30} stationary phases with a mobile phase of not less, than 97% water by volume [*Nagae*, U.S. Patent]. This patent may negate the opportunity for users to take full advantage of these long-chain phases in highly aqueous media.

2.5.2.5. WIDE PORE DIAMETER PHASES

If the pore diameter of a silica gel packing is widened, than the bonded phase is less densely packed and dewetting can be delayed. However, the lower surface area of wide-pore silicas results in lower retention, so this approach is of less interest as a means of increasing the retention of polar compounds in highly aqueous environments [*Wagrowski-Diehl et al.* 2002].

In this thesis, according to the written above, several commercial available analytical chromatographic columns were tested for the analytical performance characteristic for the analytes of interests.

2.6. CAPILLARY ELECTROPHORESIS

2.6.1. BACKGROUNDS

Even capillary electrophoresis was chosen as separation technique for identification and quantification of methimzole and its metabolites in fish whole body homogenates. CE has advantages of high separation efficiency, short analysis time, low sample and solvent consumption, low cost of the running and lower effect of matrices comparing with other separation techniques. In addition, CE can separate compounds that have been traditionally difficult to handle by HPLC for example highly polar and water soluble substances like the selected metabolites and the mother substance iself. However, there are still some barriers to overcome.

The major drawbacks in CE are the poor precision and low sensitivity in comparison to the chromatographic techniques. That could be also reason why CE is not as widespread applied technique such as GC or HPLC. Thus if these parameters are improved CE would have comparable performances as the chromatographic techniques. But to be able improve these characteristics, the theory of the separation and the instrumentation must be understood and thus they will be shortly described in this chapter.

Capillary electrophoretic separation occurs due to differences in mobility between the analytes under influence of an electrical field; some components move down the capillary faster than others. Moreover from the measured mobility physical-chemical properties of the targets like charge of the ion, diffusion coefficient or the ionization constant (pK) can be evaluated. The basic instrumental set-up, which is illustrated in figure 7, consists of a high voltage supply, fused silica capillary, buffer reservoirs, electrodes and an on-column detector.

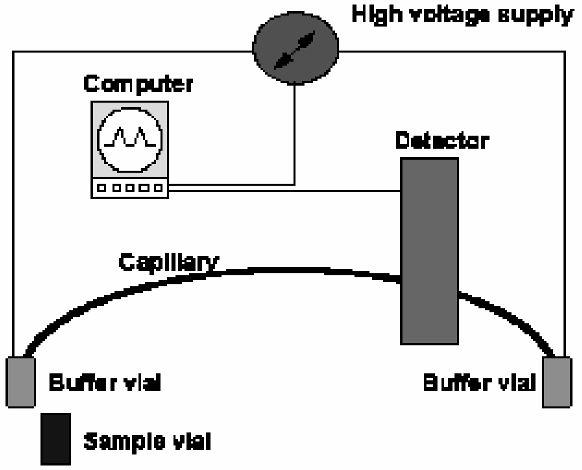


Figure 7. Scheme of a conventional CE instrument

The sample is introduced into the capillary by hydrodynamic injection or by electro-kinetic injection. The hydrodynamic injection is performed by creating a pressure difference between the buffer and the sample reservoirs during dipping the appropriate end of the separation capillary into a sample solution.

Using electro-kinetic injection, voltage is applied (1-5 kV) for a time while the beginning of capillary is inserted in the sample reservoirs. This sampling mode is at some cases more sensitive than hydrodynamic injection. However the injected amount is matrix dependent and thus application of hydrodynamic injection is more popular.

High voltage (maximum 30 kV) is generated over the capillary for the separation of analytes and the charged components move through the capillary under the electric field. Positive components migrate to the negative electrode (cathode) and negative components migrate to the positive electrode (anode) at different velocity.

The mobility difference between the separated substances depends mostly on the analyte's charge and size.

$$\mu_{ep} = \frac{Q}{6\pi\eta r} \tag{1}$$

where μ_{ep} is the electrophoretic mobility of a substance, Q is the charge of the substance, η is the viscosity of separation medium and r is the hydrodynamic radius of the substance. The charge of ion strongly depends on pH of running buffer, and thus the migration velocity of the molecules also differs with the pH. The size is a combination of the sample component and the bounded water shield to the solute. This is the reason that small inorganic metal ions migrate with similar velocity as the volatile alkyl-amines.

Modifiers like as organic solvents, surfactants, cyclodextrins or polymers can be added into the separation buffer to enhance mobility differences between the target components. When cyclodextrin is added into the buffer the technique is called electro-kinetic chromatography and when surfactants are added it is micellar electro-kinetic chromatography. In these cases the separation depends also on the solubility of a solute since more apolar ones stays longer in the micelles produced by the surfactant. However, the separation can be modified by other factors. When the capillary is filled with a solid phase the technique is classified as capillary electrochromatography (CEC).

In most applications the capillary is by fused silica. This material contains on its surface silanol groups. These groups are slightly acidic thus they are negatively charged at alkaline condition. When high voltage is generated over the capillary, the positively charged buffer constituents migrate towards the negative electrode but during the migration they also interact with the negatively charged silanol groups producing a layer which bring the neutral and negatively charged compounds towards the cathode too.

This flow is called as electro-osmotic flow (EOF) which is proportional to the dielectric constant, zeta potential at the capillary-buffer interface and inversely proportional to the viscosity of the separation medium. Thus the mobility of the solutes depends not only the μ_{ep} also on the EOF as shown in equation 2 when the separation buffer does not contain any modifiers.

$$\mu_{app} = \mu_{ep} + \mu_{eo} \quad (2)$$

where μ_{app} is the apparent mobility of the compound and μ_{eo} is the mobility of EOF. Thus EOF play a big role in the separation. Generally, EOF can not be fully controlled small changes in the EOF cause big change in the migration velocity of the solute and poor precision.

However, EOF has also advantages. On one hand, negatively charged or acidic compounds can be also separated after the detection of positively charged or basic compounds without any modification of the instrumentation. On the other hand, the flow profile is flat because the positive charge density is high close to the capillary wall and there is no drop in the capillary. This causes no peak broadening like the parabolic flow profile in HPLC and GC, and that is one of the reasons why such a high resolution can be achieved in capillary electrophoretic separations. Under typical conditions, a CE separation invokes 50,000 to 500,000 theoretical plates, which is at least one or two magnitude higher than in HPLC and better than capillary GC.

The most commercial detection mode is based on spectrophotometry (UV/VIS, DAD or fluorescence). Spectrophotometric detectors used in CE might be an order of magnitude less sensitive compared to those available for HPLC because the former detectors use shorter path length. For enhancing the sensitivity, different stacking or sweeping techniques were developed. Additionally, the detection path length can be also elongated by the use of a bubble or Zet-detector cell. However these methods to improve the method sensitivity can not be used for all cases and the enhancing factor in some cases is not enough for trace analysis. For example bubble detector cell has enhancing factor of 3-5 and Z-cell has 10-30, and peak broadening effect often occurs. Moreover, these special detector cells cannot be used with all type of instrument. Thus other techniques should be developed for decreasing the detection limit of the capillary electrophoretic separations. Another detection system has been also coupled with CE such as MS, conductivity, electrochemical or radioactive detector.

Scheme of optimisation process: since the selected analytes are Lewis acids or bases CZE or free solution CE was selected that is the simplest capillary electrophoretic technique. In CZE the separation is based on the apparent mobility of the solutes. At first, theoretical mobilities of selected analytes were calculated with a help of self-made program. The program plotted the ratio of charge and molecule mass on the two third ($Q/M^{2/3}$) in the function of pH in accordance with equation 1. From that diagram the pH range was estimated in which the analytes are fully or partly charged.

Then the buffer system of the background electrolyte (BGE) was selected. The buffer capacity should be the maximum in the estimated separation range to have similar mobility of the targets for run to run. On the other hand, the mobility of the buffer electrolyte should be in the range of the analyte's mobility to avoid the tailing of the peaks.

After selecting the buffer system, its optimal concentration had to be determined. The concentration of the buffer determines the EOF, current and peak symmetry. With increase of the concentration of running buffer the current increases since the resistance of the background electrolyte (BGE) decreases according to the Ohm equation.

After the concentration of BGE separation buffer was determined, the pH of running medium was optimized. After determining the optimal concentration and pH of the BGE the buffer system, organic modifier like acetonitrile was added into the background electrolyte at different concentration and their effect on the separation quality was investigated.

Then capillary dimension like inner diameter (I.D.) and length were optimised, finally, the effects of applied voltage and temperature on the separation and current were investigated. The final separation protocol is summarized in chapter 3.3.4.

2.6.2. EFFECTIVE MOBILITY SCALE TRANSFORMATION (IDENTIFICATION)

One major disadvantage of CE compared with HPLC is its poor reproducibility of MT and peak area. The low precision of these parameters is often related to little changes in EOF due to uncontrollable alternations of the capillary surface, which lead to MT shifting. If the effect of EOF can be eliminated on the migration of the solutes the reliability of the identification and quantification would be dramatically improved.

Thus when the migration times are transformed into effective mobility ($\mu_{eff,}$ [$cm^2/V*s$]) the method is assumed to be more representative and precise [*Schmitt-Kopplin et al.* 2001]. The effective mobilities of target substances are determined with use of the following equation.

$$\mu_{eff} = \frac{L_d * L_t * (t_{eof} - t_m)}{V * t_{eof} * t_m} \tag{3}$$

where L_d is the effective length, L_t is the total capillary length, V is the applied voltage, t_{eof} is the time of the EOF and t_m is the MT of the solute.
Effective mobility value shows the charge of the compounds: if it is anion at the separation condition, its effective mobility is negative and positive when the analyte separated as cation. Moreover, this parameter is independent on the EOF, and capillary dimensions such as the applied voltage and the length of separation capillary. When every registered point of the electropherogram is transformed to effective mobility, effective mobility scale is arisen.

The effective mobility can be normalized ($\mu_{eff,norm}$) with the following equation, when a marker with known mobility is added to the injection solution:

$$\mu_{eff,norm} = \mu_{int} + \frac{L_d * L_t * (t_m - t_{int})}{V * t_{int} * t_m} \tag{4}$$

where μ_{int} is the mobility of internal standard and is the migration time of the internal standard. Normalized effective mobility scale (normalized μ-scale) is given, when the detected signal is plotted in the function of normalized effective mobility.

Mobility scale or normalized mobility scale can be used for quantification, since the transformation from time scale does not result in any loss of information due to the bit-to-bit correspondence. On the other hand, better precision of peak area is expected when the electropherogram is transformed into mobility scale [*Schmitt-Kopplin et al.* 2001].

This transformation is not usual in other liquid phase separation technique but necessary in CE to overcome the variations of MTs and peak area. Considering the “CE-mode thinking” the measured electropherograms were transformed to effective mobility scale, and then the performance characteristics were determined and compared with the characteristics determined form the conventionally used time-scale. The transformation process was made by a program called GelTreat [*Kudryavtsev et al.* 2000]. Thus it will be shown from different examples that this scale can be easily or routinely applied for capillary electrophoretic separations.

2.7. LIQUID CHROMATOGRAPHY-Q-TOF MASS SPECTROMETRY

The mass spectrometer provides the most definitive identification of all of the HPLC detectors. It allows the molecular weight of the analyte to be determined – this is the single most discriminating piece of information that may be obtained-which, together with the structural information that may be generated, often allows an unequivocal identification to be made. The high selectivity of the mass spectrometer often provides this identification capability on chromatographically unresolved or partially resolved components.

This selectivity allows the use of isotopically labelled analytes as internal standards and this, coupled with high sensitivity, allows very accurate and precise quantitative determinations to be carried out.

The advantage of the mass spectrometer is that in many cases it can provide the absolute identification. It provides not only structural information from the molecule under investigation but it may also provide the molecular weight of the analyte. This is, in most cases, the single most important and discriminating piece of information available to the analyst which, when determined, immediately reduces, dramatically, the number of possible structures for the analyte. Another advantage of mass spectrometry is its sensitivity. A full-scan spectrum and potentially an identification can be obtained from pictogram amounts of analyte. In addition, it may be used to provide quantitative information, usually to low levels, with high accuracy and precision.

A mass spectrometer can be considered to comprise four component parts, as follows:

- a method of the sample introduction (interface)
- a method of ion production (ionisation)
- a method of ion separation (ion analyser)
- facilities for ion detection and data manipulation.

2.7.1. ELECTROSPRAY IONISATION

A liquid, in which the analytes have been dissolved, is passed through a capillary, at atmospheric pressure, maintained at high voltage (typically 3-5 kV for the positive ions). This high voltage disperses the liquid stream, forming a mist of highly charged droplets that undergo desolvation during their passage across the source of the mass spectrometer. As the size of the droplets reduces, a point is reached (within 100 µs) at which the repulsive forces between charges on the surface of the droplets are sufficient to overcome the cohesive forces of surface tension. A Coulombic explosion then occurs, producing a number of smaller droplets (Figure 8) with a radius approximately 10% of that of the parent droplet. A series of such explosions then take place until a point is reached at which ions of the appropriate analytes dissolved in these droplets are produced and are transferred through a series of focusing devices (lenses) into the mass spectrometer. There are two alternatively explanations for the actual mechanism by which these ions are produced: the ion evaporation [*Iribarne & Thompson* 1976] and the charge-residue [*Schmeizeisen-Redeker et al.* 1989] models. According to the ion-evaporation model, the droplets become smaller until a point is reached at which the surface charge is sufficiently high for direct ion evaporation in to the gas phase to occur. In the case of charge-residue model, repeated Coulombic explosions take place until droplets are formed that contain a single ion. Evaporation of the solvent continues until an ion is formed in the vapour phase.

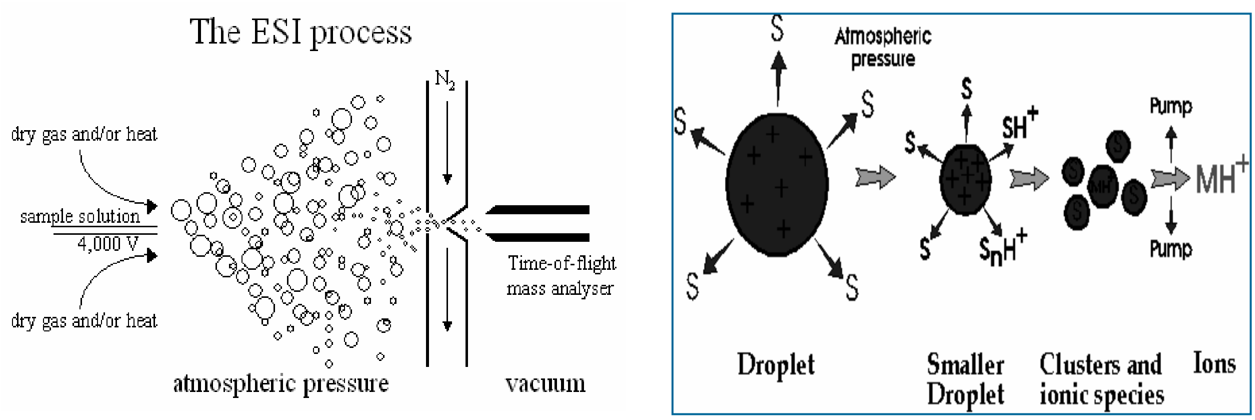


Figure 8. Electrospray ionisation process and mechanism of the ion formation

The flow rate of liquid in the HPLC-electrospray system is paramount in determining performance both from chromatographic and mass spectrometric perspectives. The flow rate affects both size and size distribution of the droplets formed during the elctrospray process and the number of charges on each droplet. This has an effect on the appearance of the mass spectrum which is generated. It should be noted, that the smaller the diameter of the spraying capillary, then the narrower the droplet size distribution and the more efficient the transfer of sample to the mass spectrometer. The electrospray is most efficient at flow rates between 5 and 10 µl/min, which are not directly compatible with the commercial HPLC applications (equipments). There are two approaches to providing reduced flow rates of an appropriate magnitude:

- the usage of HPLC columns with reduced diameters (microbore columns)
- or splitting the flow from a conventional column.

2.7.2. ION ANALYSER:TOF

In some aspects, the time of flight analyser is the simplest of the mass separation devices. This system relies on the fact that if all of the ions produced in the source of a mass spectrometer, by whatever technique, are given the same kinetic energy then the velocity of each will be inversely proportional to the square root of its mass. As a consequence, the time taken for them to traverse a field-free region will be related in the same way to the m/z of the ion. A complete mass spectrum is obtained simply by allowing sufficient time for all of the ions of interest to reach the detector. In the TOF instrument it is essential that ions of all m/z ratios present in the source are transferred, simultaneously and instantaneously, into the mass analyser at a known time so that their times of flight, and thus their m/z ratios, may be determined accurately. Were ions to be introduced continuously it would be impossible to determine exactly, when each began its passage through the flight tube and therefore to calculate its m/z ratio. A complete mas spectrum at a specific time is therefore obtained and when this has been recorded, a matter of milliseconds later, a further set of ions can be transferred from the source. Fast scanning, only limited by the time it takes the heaviest ion to travel from the source to the detector, is possible and any distortion of ion intensity brought about by changes in analyte concentration during the scanning process is removed.

The resolution of the TOF analyser is dependent upon the ability to measure the very small differences in tim e required for ions of a similar m/z to reach the detector. Increasing the distance that the ions travel between the source and the detector (increasing the length of the flight tube) would accentuate any such small time-differences. The implication of such an increase is that the instrument would be physically larger and this goes against the current miniaturising trends of all analytical instruments. A solution to use one or more ion mirrors is known reflectron. This reflect the ion beam, in the case of an instrument with a single reflectron, towards a detector located at the same end of the instrument as the source. The distance that the ion travels is thus doubled without an equivalent increase in the length of the flight tube. Some instruments now incorporate more than one reflectron, so increasing the distance travelled by the ions even more and this way the resolution of current TOF instruments has been increased to more than 10000. The advantage of the TOF instrument, in addition to its simplicity, is its fast scanning capability and for this reason it is increasingly being encountered in LC-MS instrumentation, particularly when fast analysis or high chromatographic resolution is involved.

2.7.3. Q-TOF TANDEM MS-MS

It is unlikely that the molecular weight alone will allow a structural assignment to be made and it is therefore desirable to be able to generate structural information from such techniques. Tandem mass spectrometry is a term which covers a number of techniques in which one stage of mass spectrometry is used to isolate an ion of interest and a second stage is then used to probe the relationship of this ion with others from which it may have been generated or which it may generate on decomposition.

There are a large number of different MS-MS experiments that can be carried out [*McLafferty* 1983; *Busch* 1988].

In the Q-TOF instrument, the final stage of the triple quadropole is replaced by an orthogonal TOF analyser as figure 9 shows.

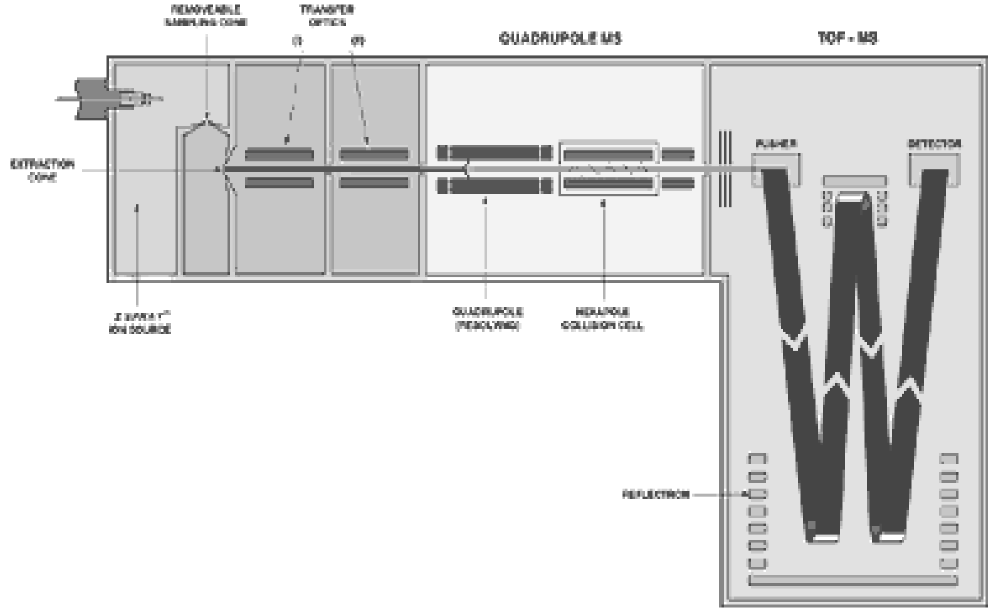


Figure 9. The construction of a Q-TOF instrument. TOF is here equipped with three reflectrons.

This configuration is typical for the latest generation of TOF instruments in which a number of reflectrons are used to increased the flight path of the ions and thus increase the resolution that may be achieved. The difference between this and other MS-MS instruments is the way in which the MS2 unit operates. To reiterate, in contrast to other mass analysers which are scanned sequentially through the m/z range of interest and provide MS-MS spectra of user selected masses, the TOF analyser detects all of the ions enter it at a specific time. It is therefore possible, particularly in view of the high-scan-speed capability of this instrument, to provide, continuously, a full MS-MS product ion spectrum of each ion produced in the source of the mass spectrometer. The disadvantage of this mode of operation is that it renders the Q-TOF system unable to carry out precursor and constant-neutral loss scans.

2.8. SAMPLE PREPARATION METHODS

The reason why there is such a need for improvement in sample preparation techniques is that the majority of the sample analysis time is spent in preparing the sample. One study [*Pipkin* 1990] showed, that more than 60% of analysis time was spent in the sample preparation to only about 7% for the actual measurement of the sample constituents. It is then clear, that analytical laboratories are present-day concerned more and more with speed and efficiency as well as reliability. An effective, analyte specific sample pre-treatment method is indispensable to a selective, sensitive and accurate analytical measurement. In addition, sample preparation is not only for achieving more efficiency on the analytical method, but it provides even a powerful tools in the hand of the analytical chemists to make certain a good maintenance of the analytical equipment that is applied. The two major goals of a sample pre-treatment method are: to clean up the sample (removing the potentially high number of contaminants and disturbing substances) and to increase the concentration of the target analyte(s) in the mixture (enrichment). It is evident, that the sample preparation must be as simple as it can without any loss on efficiency.

Various techniques are known for the adequate pre-treatment of the wide variety of sample matrices for the analytical measurement. In the past liquid-liquid extraction has played a major role, but the popularity of the solid-phase extraction is growing at a fast rate in the recent years.

In SPE solutes are extracted from a liquid phase into a solid phase, which consists of small, porous particles of silica with bonded organic phase or of an organic polymer. SPE is not limited to the use of solid particles to extract solutes from a liquid sample; air or other gaseous samples can also be passed through a packed tube.

SPE is similar to chromatographic processes, but only in the basics: both use a small tube(column) packed with solids that acts as stationary phase. In SPE the sample solution is the mobile phase. Multiple equilibria for the various analytes exist between the liquid and solid phase. These equilibria can perhaps be best expressed in terms of the capacity factors of the various sample components. Ideally the capacity factor of the sample components will be very high, about 100-1000 or more. Under these conditions the sample components will be retained as a simple, tight band on the column. After a brief rinse, theretained materials are eluted rapidly as a group under conditions where the capacity factors will be as low as possible. There is ordinarily no separation of individual analytes in SPE. All the compounds are retained as a sharp band on the solid phase and then are eluted together by an appropriate liquid solvent. Table 7 gives a short overview of the LLE and SPE methods compared to the liquid chromatographic technique.

Table 7. Comparision of the liquid-liquid and solid phase extraction methods with the liquid chromatography

	LLE	SPE	LC
Extractive phase	liq. emulsion	porus (monolith) solid	porous solid
Typical sample	1-100 ml or more (aq.)	1-100 ml aq.(org.)	Aq.-organic or organic
Partition equilibrium	single	multiple	multiple
Separation of individual analytes	no	no	yes
Automatisation	no	yes	no
Elution from extractive phase	Seldom needed; H2O, pH control	Organic liquid	H2O-organic liq. Mixture
Concentration achived	moderate	high	slight

The solid phase extraction has several important advantages over liquid-liquid extraction: faster, easier, it can be automated, needed much lower organic solvent consumption (lower time consuming, and lower costs) [*McDowall* 1989; *Majors* 1991]. SPE shows less stringent requirements for the separation and higher concentration factors can be achieved with it.

The four main steps of an SPE extraction [*Fritz* 1999] are:

1. Conditioning. Before adsorption of analytes by the solid phase can begin, the sorbent bed must be prepared and made compatible with the liquid solution. The necessary pre-treatment

involves the use of a mediating solvent that will promote better surface contact between the phases.

2. Adsorption.

3. Washing. A carefully chosen wash liquid affords the opportunity to remove co-adsorbed matrix materials from the SPE column. What constitutes the right choice of a wash solution is difficult and depends strictly upon the actually tasks. Of course, the washing solution must not contain a percentage of organic solvent high enough to partially elute the sample analyte.

4. Elution. In this step the adsorbed analytes are removed from the sorbent surface. The eluting solvent must be chosen that it elutes the analytes completely from the sorbent. In terms of capacity factor, this means that k of the analytes should be as near as possible to zero. It is required in several cases, to change the eluting solvent to a weaker liquid that is better suitable for the further analytical measurement. This change in solvent quality is carried out predominantly by evaporating the elution solvent and replace it with solvent adequate for the analytical method. It renders even a concentration of the analyte in the solution.

The point at which the first analyte leaves the packed tube is called breakthrough volume (VB). The VB is sometimes taken as the volume when the concentration of the analyte leaving the tubes constitutes 1% of its initial concentration in the sample. Wells defined the breakthrough volume as the maximume sample volume from which 100% recovery can be achieved [*Wells* 1985]. Concentration factor is the ratio of the sample volume to the volume of the solvent used in the final re-dissolving step.

Various SPE techniques are in use. A short overview is to be read below.

2.8.1. NP-SPE

Normal phase SPE polar solid particles (SiOH, FL, Diol, NH2, NO2, CN) are then used to extract polar substances. Relatively strong (2-10 kcal/mol) interactions such as dipole-dipole and hydrogen bonding can be occur between the extracted molecule and the active groups of the surface. The extracted substances are finally eluted by a polar solvent. They retain hydrophilic organic material much more strongly than do compounds that are more hydrophobic. Silica gels and florisil are extensively used in clean up of samples prior to analysis. Florisil is a magnesic silica gel that can be activated by heating up to 650°C. It is widely used to clean up liquid organic solvent extracts of plant and animal tissues.

The goal in RP-SPE is to isolate relatively non-polar analytes from a polar sample such as water. This type of application requires the use of relatively hydrophobic adsorbent particles, such as silica with bonded C4-C18 groups or an organic polymer with benzene rings. Polymer solvents are highly hydrophobic and so they are more retentive, and have greater surface area then the bonded silica phases, and they are applicable in the whole pH range opposite to the limited pH usage of the silica phases. Typical interactions are weak van der Waals interactions. The disadvantage of the polymer based materials to the bonded phases are, that they are less selective and retain polar substances with lower efficiency, however they retain chemicals in their ionised form more efficient [*Pichon et al.* 1996]. Extracted substances are eluted by a small volume of an organic solvent.

IP-SPE phases contain particles with ionic exchange groups on the surface. IP-SPE cartridges are used for extraction ionic analytes or analytes that can be converted to ionic forms by adjusting the sample pH. Substances are retained by strong electrostatic interaction and can be eluted with an organic or inorganic solvent converting then back to the molecular form or can be removed by elution with a solvent containing a relatively high concentration of a displacing ion.

2.8.2. POROUS GRAPHITIC CARBON

The porous graphitic carbon sorbents can be arranged as: graphitic carbon blacks (GCB) and porous graphitic carbons (PGC). However, they are the most hydrophobic phases on the market at the moment, they are suitable for the extraction of very polar, extremely water soluble compounds. The polar adsorption arises from surface oxygen complexes that are few in the number but interact strongly with polar compounds. Only difficulty is, that they are hardly to elute from the cartridge. The application field of these sorbents are simultaneous extraction of neutral, basic and acidic compounds [*Jennion et al.* 1999; *Matisova et al.* 1995*; Hennion* 2000; *Dicoria et al.,* 1993].

Both GCBs and PGCs behave, in addition, as non-specific sorbents via van der Waals interactions and even as anionic exchange sorbents. While GCBs do not have micropores (<20Å), PGCs contain macropores and are more highly homogenous hydrophobic surfaces.

2.8.3. MATRIX SOLID PHASE DISPERSION (MSPD)

The MSPD technique was originally developed for residue determination in pesticide analysis [*Sherma* 1997; *Viana et al.* 1999; *Lott and Barker,* 1993].0.1-5 g homogenised (blended) sample is mixed with the solid phase which is usually florisil, silica or aluminium-oxid. This affords a better contact between the extraction phase and the extractant sample. The mixture is then filed into a glass column which is cased with silanised glass-whool. Analytes will be then eluted with high amount of organic solvents. This extraction and clean up in one step method showes good reproducibility and recovery rates. However, applications for animal tissue samples were not published before with this method, we investigated its capability for the complex fish matrix. Advantageous is, that the composition of the extraction solvent can be altered in accordance to the analytical requirements.

2.8.4. SAMPLE PRE-TREATMENT FOR BIOLOGICAL SAMPLES

Isolation of drugs, drug metabolites and other relatively small organic substances from biological samples is complicated by the presence of proteins and other large molecules in the samples. Protein removal by various precipitating reagents has the disadvantage that some relevant substances may be removed by occlusion. Traditionally, LLE has been used in most clinical/toxicological laboratories. However, SPE is gaining in popularity for removal and isolation of drugs and other substances from biological samples. Given the low concentration of drugs and so an in biofluids and the presence of many interfering substances in the sample matrix, an extraction – concentration step is always required. While SPE is attractive for this, also our applications were tended this direction.

For developing of an analytical method, spiked samples are used to check the analytical efficiency. US-EPA defines extraction efficiencies as acceptable in the range between 70-120% for matrix samples.

2.9. DETERMINATION OF ANALYTICAL PERFORMANCE CHARACTERISTICS

The concentrations of the target compounds were calculated from electronically integrated peak areas using external calibration curve. The integration was made by using self-developed program called GelTreat for the capillary electrophoresis measurements and by the HPLC system control software Turbochrom 3 and MassLynx 4.0 for the HPLC measurements. For calibration the integrated peak areas were plotted in a function of the theoretical concentration of standard solution.

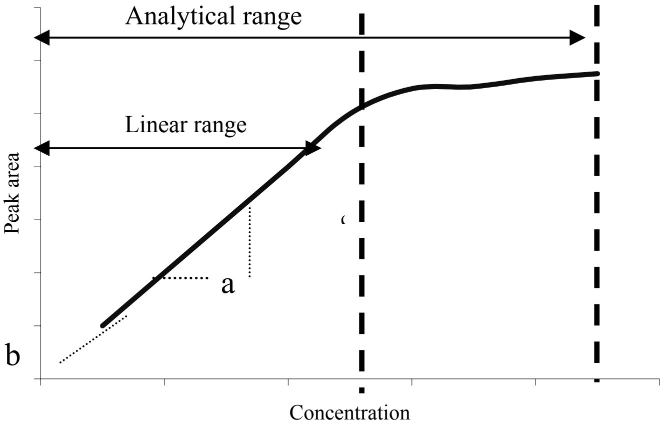


Figure 10. Peak area response in function of analyte concentration

As shown in figure 10, peak area is proportional to analyte concentration at low range of the measured concentration. The linearity of peak area response is determined by regression coefficient with use of equation 5.

$$r^2 = \left(\frac{\Sigma(c_i - \bar{c}) * (A_i - \bar{A})}{\sqrt{\Sigma(c_i - \bar{c})^2 * (A_i - \bar{A})^2}}\right)^2 \tag{5}$$

where c_i is the actual concentration of the target compounds, $\bar{c}$ is the averaged concentration, A_i is the peak area at actual concentration of target compounds and A is the averaged peak area. When the regression coefficient is higher than 0.99, linear dependency can be concluded and thus calibration can be used for determination of concentration of analytes from the integrated peak area with use of the following equation.

$$c = \frac{A - b}{a} \tag{6}$$

where A is the peak area, a is the slope of the linear slope, c is the concentration of the analyte and b is the y intercept of the trend line (see figure 10). The value of the slope and intercept of y-axis for each analytes should be determined by separating the analytes at minimum five different concentrations every measuring day.

The required analytical performance characteristics for validation were determined according to the standards DIN EN 482, EN 4500 and ISO/IEC Guide 25 to characterise the correctness and precision of the optimised method.

Resolution: This performance characteristic shows if the solutes are separated for reliable identification. The resolution (R_s) can be defined as a following:

$$R_S = 2 * \frac{t_{m,2} - t_{m,1}}{w_1 + w_2} \qquad (7)$$

where $t_{m,I}$ is the identification parameter of compound i, and w_i is the peak width of compound i. If the R_s of the separated substances higher than 1.5, they can be differentiated from each other when they present in the sample.

Precision: The parameter describing the precision is the standard deviation (s^2) which was calculated with the equation 8.

$$s^2 = \sqrt{\frac{\Sigma(x_i - \overline{x})}{n - 1}} \qquad (8)$$

where x_i is the measured data, $\overline{x}$ is the average and n is the number of repetitions. For precision of identification and quantification the measurements were repeated three or five times at three different concentrations in the working range.

When the relative standard deviation (RSD) of identification parameter were determined between 1-2%, the method is concluded enough precise to use for real sample analysis. When the RSDs of peak areas that describe the repeatability of quantification were lower than 10% precision fits to the specific analytical requirement.

Linearity and working range: standard solutions at different concentrations were measured and the integrated peak areas were plotted in the function of the solution concentration. Trend line was drawn through the measured points and the correlation coefficient was determined to check the proportional response between peak area and analyte concentration.

Sensitivity: The limit of detection (LOD) and limit of quantification (LOQ) show the sensitivity of the method. They were calculated according to [DIN 32645] after calibration. LOD and LOQ are usually defined as the concentration at which the peak area three and ten times bigger, than the noise one (see equation 9).

$$\mathrm{LOD} = \frac{3 * \mathrm{noise}}{a} \qquad \mathrm{LOQ} = \frac{10 * \mathrm{noise}}{a} \qquad (9)$$

where noise is the averaged peak area of the noise in the baseline and a is the slope of the calibration curve.

Accuracy: The accuracy of the whole analytical procedure including the sampling, sample preparation and separation was characterized by the recovery and shows the correctness of quantification. The relative recovery (w) is defined as:

$$w = \frac{c_{theo} - c_{meas}}{c_{theo}} * 100\,\% \qquad (10)$$

where c_{theo} is the theoretical concentration, c_{meas} is the quantified concentration calculated from calibration curve.

When the recovery of the measured substance is 100%, the measured concentration is identical to the theoretical one. The recoveries were determined always for the overall procedure at three different concentrations of solutes. If the relative difference between the measured and theoretical amount was lower than 10% the method was correct for sample analysis.

Thus for having a reliable method that can be used for measuring unknown samples the performance characteristics which are selectivity, linearity, sensitivity, precision and accuracy were determined including the whole analytical process.

3. RESULTS AND DISCUSSION

3.1. PREDICTION OF CHROMATOGRAPHIC BEHAVIOUR OF THE ANALYTES

It is essential in the chromatographic practice for the planning of separation to know about all physical and chemical properties of the analytes. From chromatographic view, the hydrophobic- hydrophilic characteristic is one of the most important information about the probable behaviour of the analytes on the analytical columns or sample preparation cartridges and also in the case of any other extraction methods. Abilities for ionisation, protonisation or binding potential of the functional groups on the substances define the changes in the molecular structure and so the solubility in aqueous or organic solvents at the time. This solubility values have influence on the participation processes between stationary and mobile phases, thus determined the retention of the analytes. Most important values, which determine the sorptic-adsorptic processes are pKa, log P and log D values. The pKa which derives from the negative logarithm of the acidic dissociation constant (K_a) represents the ionisation ability of the ionisable groups of the target analyte. The logP values define the logarithm of the octanol-water partition coefficient and thus the hydrophobicity of compounds. The logD represents the logarithm of the distribution coefficient, what is similar to logP with the extended meaning and information about distribution forces between other solutions than octanol and water. Log D is the most interesting parameter from the standpoint of the planning of the chromatographic separation.

Partition coefficients can be measured by several techniques, ranging from shake flask technique to chromatographic methods. However, experimental determination of partition coefficient is time and material consuming. Accordingly, numerous theoretical methods have been devised allowing increasingly accurate prediction of log P values. We present two computer software based on theoretical calculation, which we had used for the prediction of relevant physical- chemical parameters for the planning of the separation method and sample preparation. The software Pallas is a trade mark ware and not available without buying the software pocket, while Sparc is a free for commercial use available freeware on the internet. We introduced both programmes for prediction

The log P values were determined by the software packet Pallas3.0. The values are determined from the data in the fragment databases. The Prolog P 5.1 modul uses for the prediction three different fragment database allowing different methods to calculate $\mathrm{kogP}_{\mathrm{octanol}}$ These are CDI-Rekker, Atomic and Atomic 5, which use different fragmental systems approaches. CDI-Rekker database is based on Rekker´s collection of hydrophobic fragmental constants [*Rekker* 1979] derived from an extended set of about 1000 logP values in the octanol-water system. Using Rekker´s fragmental method a partition coefficient is obtained from

$$\log P = \sum a \ f_i + \sum b_j F_j \qquad (11)$$

where a_i is the incidence of fragment i

b_j is the incidence of correction term j

F_j is a multiple of a constant term.

In the Atomic database log P is calculated using atomic fragments. The body of these fragments is only one atom. The fragment is then described by what it is adjacent to. The atom types used in this system are described in the paper of Broto et al., in which is introduced a system based on atomic contributions instead of using fragments of multiple atoms. The equation is

$$\log P = \sum n_i a_i \qquad (12)$$

ni is the number of atoms of type i

ai is the contribution for atom type i

It is subdivides the atoms C, O, N, S, Cl, Br, I, F into 221 types according to their neighbours, their bonds and the bonds of their neighbours. The Atomic 5 database uses atomic fragments based on the work of Ghose et al.

The logP calculation involves five major steps, as follows:

- The structure of the molecule is decomposed into fragments from the current fragment database. In this phase fragments are checked, one by one, for their presence in the molecule.
- If more than one possible decomposition of the input molecule is found, the chemically most reasonable one is selected for further evaluation. According to the basic principles of the fragmental methods of logP calculation, this is postulated to be the decomposition invoking the fewest fragments.
- The program explores all possible interactions between different parts of the molecule. In the case of Rekker´s method, the proximity effects of polar fragments are determined by locating all polar fragments at appropriate distances from each other.
- The logP of the molecule is calculated as the sum of the contributions of the fragments and the interactions.
- Finally the combined logP value is preticted as the weighted average of the values obtained according to the following equation:

$$\log P_{combined} = \sum_{i=1}^{n} w_i \log P_i \qquad (13)$$

where w_i is weight of database i

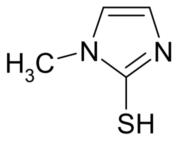
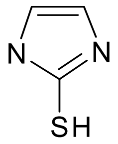
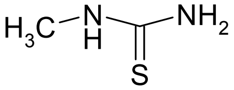
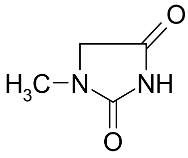
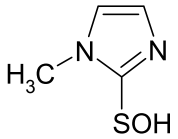
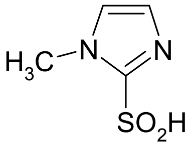
$logP_i$ is the logP value predicted using database i

Another object of the investigation was, prediction of the pKa and logD values by a commercial available free software on the internet. Freeware Sparc is available at the internet [Sparc].

3.1.1. PREDICTIONS BY PALLAS

Results of the prediction are shown on the next pages. The theory of the fragment based prediction were detailed chapter 3.1.

Table 8. Predicted pKa values of the methimazole and its metabolites. Predicted by Pallas

Substance	Predicted pKa values	
	Acidic groups	Basic groups
 MET	none	-N=: 4.41
 SHMET	-SH: 12.19	-N=: 4.36
 MTU	none	-NH2: 14.39
 MEH	-NH-: 9.28	none
 MSOH	none	-N=: 6.37
 MSO2H	-SO2H: 1.38	-N=: 7.09

It can be read out from the predicted values, that all the analytes of interest are very polar. All of them contain possible ionisable groups, which affects the chromatographic behaviour of them. Calculated pKa value of methimazole predicts a basic characteristic on the nitrogen and a protonated form of it under pH 2.4 and an unprotonated form below pH 6.5. pKa value of MTU foreshadows a strong basic substance until pH 12.4. SHMET seems to be basic and acidic: it is protonated on nitrogen under pH 2.3 and dissociated below 6.4 and the acidic nitrogen is dissociated about pH 14, while MEH is 100% negatively charged on the nitrogen below pH 11.3. MSO2H behaviour accords to the acidic character of the sulphate group, which is deprotonised in the whole pH range, while MSOH showed only basic character according to the prediction.

Log P (see table below) values were determined as 1.05 for SHMET, 1.25 for MET, -0.98 for MTU, -1.22 for MEH, 0.97 for MSOH and -0.82 for MSO2H, which show an unambiguous polar character of the metabolites.

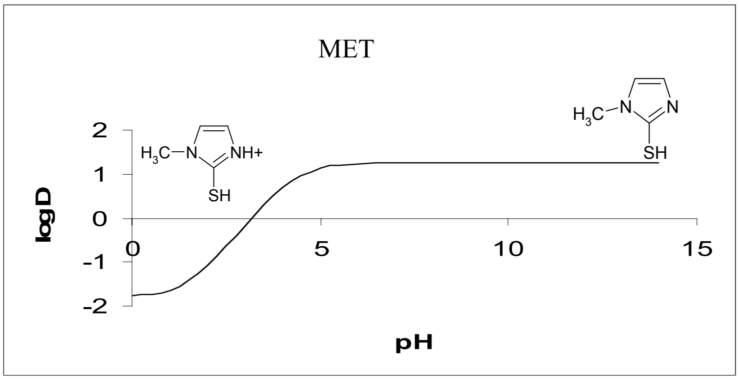
LOG P VALUES

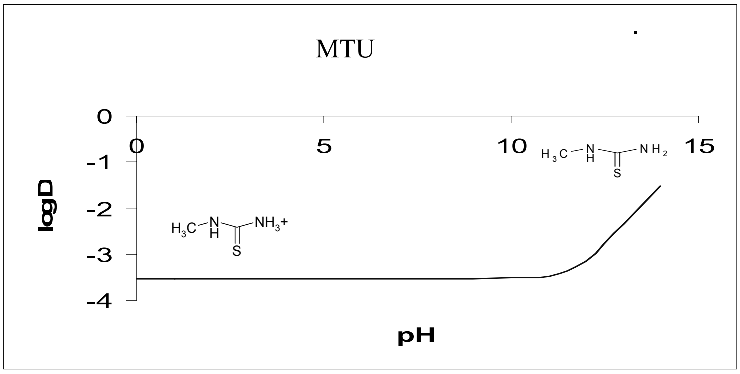
Table 9. log P values of methimazole and metabolites predicted by Pallas

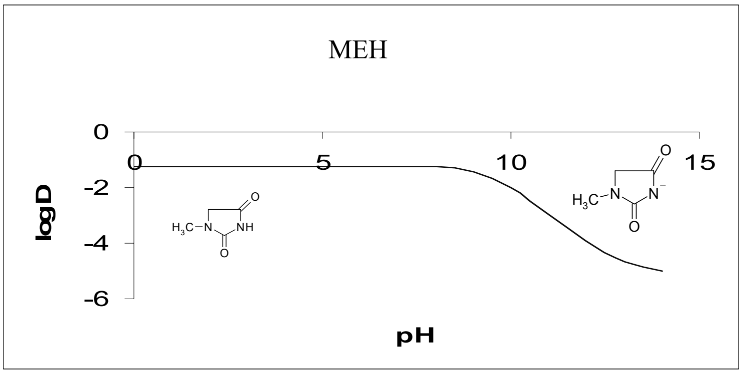
Substance	Predicted log P values
MET	1.25
SHMET	1.05
MTU	-0.98
MEH	-1.22
MSOH	0.97
MSO2H	-0.82

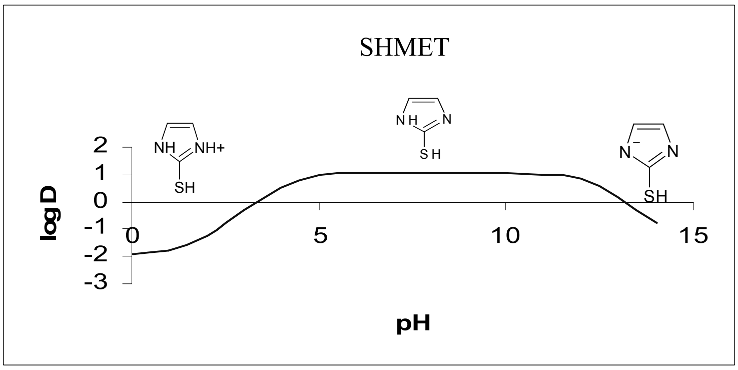
LOG D VALUES

The predicted logarithmic distribution coefficient graphs are shown below (Fig. 11.):


MET
log D
pH
2
1
0
-1
-2
0
5
10
15
H_3C–N NH+ SH
H_3C–N N SH


MTU
log D
pH
0
-1
-2
-3
-4
0
5
10
15
H_3C–NH–C(=S)–NH_3+
H_3C–NH–C(=S)–NH_2


MEH
log D
pH
0
-2
-4
-6
0
5
10
15
H_3C–N NH O O
H_3C–N N⁻ O O


SHMET
log D
pH
2
1
0
-1
-2
-3
0
5
10
15
NH NH+ SH
NH N SH
N⁻ N SH

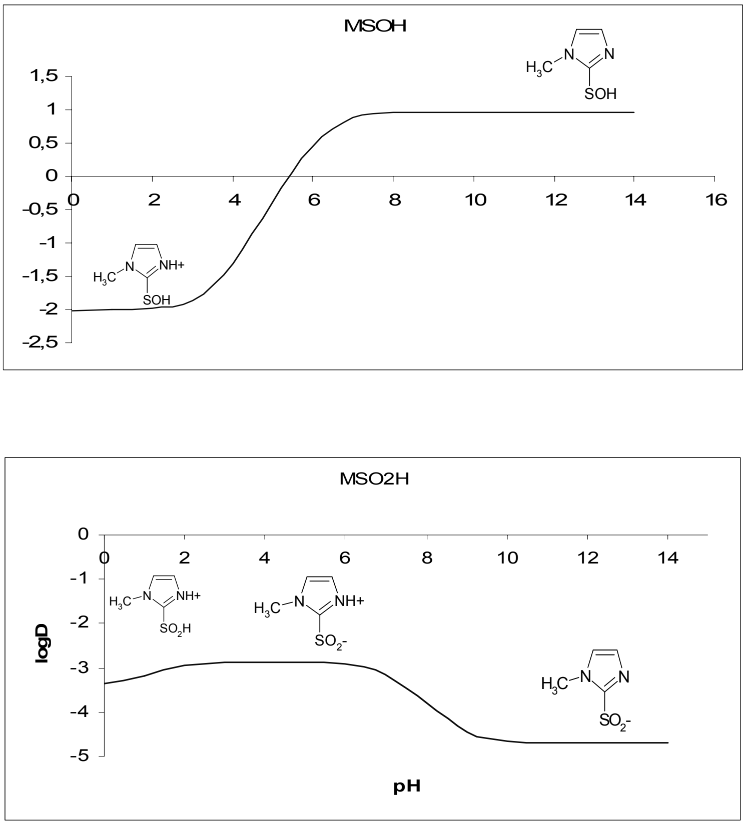


Figure 11. Pallas predicted log D curves with the predicted structural forms of methimazole and its metabolites

Calculated distribution curves predict a good and robust (pH) separation of the substances between the whole working range of the available analytical columns. Exceptions are the points of interception of two plots, e.g. at pH 4.2 MTU and MEH , at pH 7.6 MTU and MSO2H can eventually not be separated with the required resolution factor.

Predictions expressed a very high polar character for the MEH, MSO2H and MTU, while hydrophobicity of the other high polar substances can be lowered by adjusting eluent pH values.

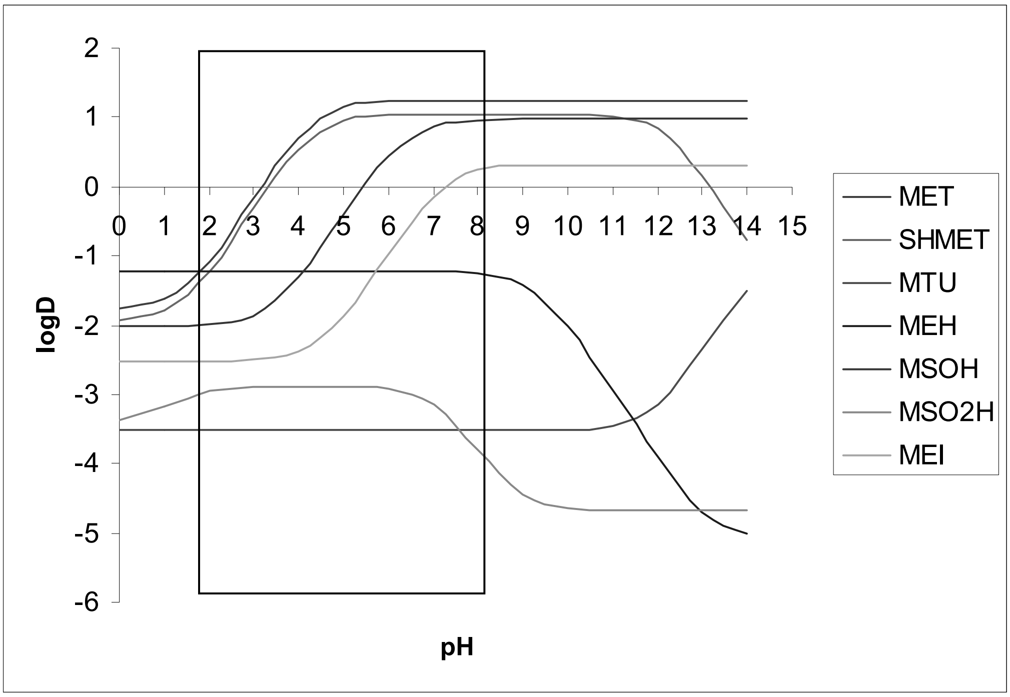
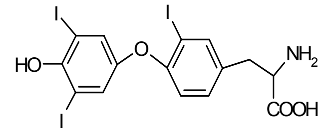
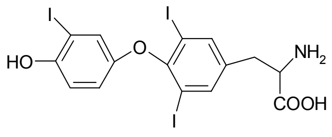
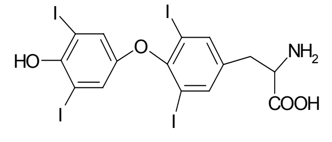


Figure 12. Predicted log D curves of methimazole and its metabolites. It was predicted by Pallas. Frame represents the working range of the used analytical columns.

PREDICTIONS FOR THYROID HORMONES

Table 10. Predicted pKa values of the thyroid hormones. Predicted by Pallas

Name (Zeichen)	Struktur	pKa mit Pallas		
		$-NH_2$	-COOH	-OH
3,3`,5`-Triiodo-Thyronine (rT3)		8.61	1.76	7.75
3,5,3`-Triiodo-Thyronine (T3)		8.22	1.44	9.08
3,5,3`,5`-Tetraiodo-Thyronine (T4)		8.26	1.38	7.67

Results of thyroid hormone prediction are seen in the table 10. As mentioned, the most ionisable groups of the substances are the carboxylic and amino groups and even phenolic hydroxyl is predicted to be dissociated at higher pH values than 10. On the basis of predictions the dissociation-association properties thus conformation appearance of the thyroid hormones and their precursors are similar and easily can be discussed: below pH 1.5 the amino groups of all substances are positively charged and all phenolic hydroxyls are in associated form. The carboxylic function groups are about 50% in dissociated form but the rate of dissociation can not be exactly defined in the case of the common of the molecules, so thus these pH values are critical from the chemical behaviour of the compounds. At very low pH values they appeare in the positively charged form due the ionisation of amino groups and unionisation of the hydroxyl and carboxyl groups of the aminoacids. At higher acidic pH values (1.5-3.5) the carboxylic group is in charged and uncharged state depending on the individual of the molecules, while amino groups are clear in positively charged and hydroxyls in uncharged modus. This range is not really desired during separation or sample pre-treatment, since the polar-apolar character of the substances are very instabil in this range. At pH values between 4 and 6 all carboxylic groups are negatively and all amino groups positively charged, while the most phenolic hydroxyls in uncharged form, so this pH range seems to be optimal for planning apolar interactions between target substances and stationary phases or other medium used for any preparation step with the analytes of interest. The isoelectric points (pI values) of the target compounds are supposedly found in this range. In the pH range between 6 and 11 carboxylic groups are in totally dissociated forms, while amino and hydroxyl groups depending on pH values in dissociated or associated forms. At pH values where amino groups are charged but hydroxyls not (pH~6), the netto charge of the molecules are about zero and this range belongs to the possible isoelectric points too. In the range pH 6 and 11 the dissociation of the hydroxyl and amino groups determine the polarity of the substances. At pH values above ~11 negatively charged hydroxyl and carboxyl groups determine the clear polar character of all analytes of the interest.

LOG P VALUES

Table 11. Predicted log P values of the thyroid hormones. Predicted by Pallas

Substance	**Calculated log P value**
MIT	1.69
DIT	3.09
rT3	6.04
T3	6.04
T4	7.44

Log P (octanol/water) calculations prove a mostly apolar character of the target compounds.

LOG D VALUES

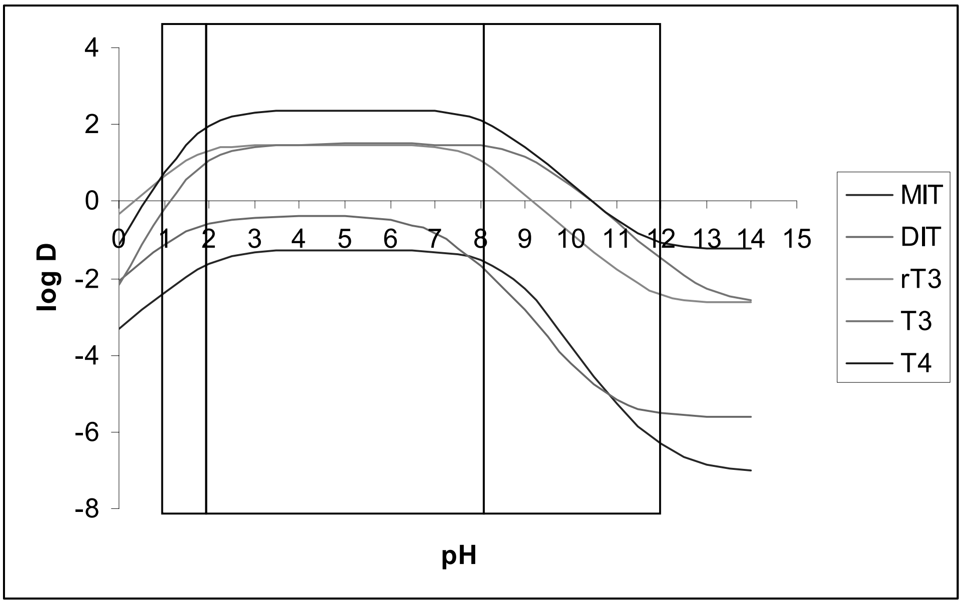


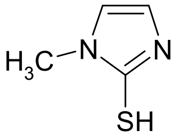
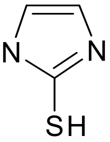
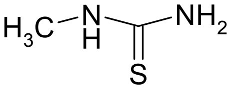
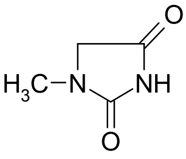
Figure 13. Predicted log D curves of the thyroid hormones. MIT and DIT are precursors of the thyroid hormones. Frames represent the working pH range of the used chromatographic columns. XTerra MS C18 column had an extended pH (1-12) range.

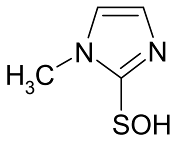
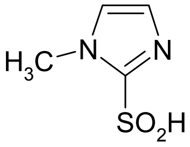
Log D predictions flatter with good separation perspectives of four substances in the pH range between 2 and 8 what is the normal operation range of the most available chromatographic columns on the market. Although T3 and rT3 are not really separated below pH 7, it seems to arrange at ph 2 or between pH 7.5-9.5. Crossing curves T3 and T4 between pHs 9.5-11.5 and curves MIT and DIT at pH ~8 and 10.5 provide unsatisfactory resolution of the named compounds. Separation of the five analytes within a single run with resolution higher than 1.5 seems to be achieved with an extended pH operation range column (s. chapter 3.2.1.) at pH ranges between 1.3-2.5, 7-7.6 and 8-10. The high log D values of rT3, T3 and T4 prognosticate a sufficient capacity factor on reversed phase columns. In the case of MIT and DIT the calculated values are squarely for a low retention of the compounds and so the extremely low and high pH ranges like pH<2 and pH>9. On the basis of the predictions these pH ranges are to be avoided, albeit accepted resolution factors can be only achieved in most of this range.

3.2. PREDICTIONS BY SPARC

PKA VALUES

Table 12. Predicted pKa values of methimazole and its metabolites. Prediction by Sparc

Substance	Predicted pKa values (Single calculation))	
	Acidic groups	Basic groups
 MET	-SH: 3.04	-N=: 5.91 H3C-N=: -0.27
 SHMET	-SH: 3.04	-N=: 6.56 H3C-N=: -1.15
 MTU	none	-NH2: 2.67 -NH=: 2.79
 MEH	none	-NH-: -1.19 H3C-N=: -0.94

 MSOH	-SOH: 9.72	Me-N=: -0.01 -N=: 7.44
 MSO2H	-SO2H: 10.6	Me-N=: -0.01 -N=: 7.42

LOG D

Sparc provided options for prediction log D values for different organic solvent to water ratios. This function is unique, so we publish the prediction results thus it is a great help to orient the solubility character of the substances of the interest. Unfortunately the freeware is not able to calculate and to draw out log D versus pH curves as does the Pallas.

Table 13. Predicted log D values of methimazole and metabolites by Sparc. The solvents were free to choose.

Solvent x /water	MET	SHMET	MTU	MEH	MSOH	MSO2H
Octanol (= log P)	0.22	-0.32	-0.32	-1.17	0.62	0.40
Acetone	1.98	1.91	1.14	0.13	1.76	1.37
Acetonitrile	1.94	1.89	0.62	0.23	1.85	1.48
Ethylacetate	1.54	1.02	0.67	-0.28	0.36	0.72
Methanol	1.33	1.61	0.26	-0.11	1.89	1.25
Generic organic	-0.41	-0.77	-0.83	-1.78	0.005	-0.24
chloroform	0.56	-1.26	-0.45	-0.61	-1.71	-0.05
dichloroethane	0.14	-1.62	-0.83	-1.03	-2.15	-0.52

THYROID HORMONES

Table 14. Predicted pKa values of the thyroid hormones. Prediction by Sparc

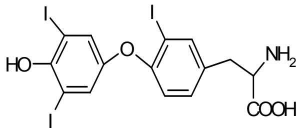
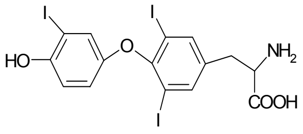
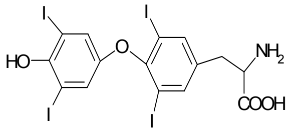
Name (Abbrevation)	Struktur	*pKa calculated with Sparc*		
		-NH$_2$	*-COOH*	*-OH*
3,3`,5`-Triiodo-Thyronine (rT3)		*6.25*	*3.66*	*5.76*
3,5,3`-Triiodo-Thyronine (T3)		*5.96*	*3.58*	*7.39*
3,5,3`,5`-Tetraiodo-Thyronine (T4)		*5.84*	*3.55*	*5.71*

Table 15. Predicted log D values of the thyroid hormones by Sparc.

Solvent x /water	T3	T4
Octanol (= log P)	6.0613	7.4524
Acetone	6.7613	8.6024
Acetonitrile	4.9457	6.8280
Ethylacetate	6.0138	8.4934
Methanol	4.6802	5.7560
Generic organic	5.5643	7.2615
Chloroform	4.2958	7.8513
Dichloroethane	3.7086	7.2483

Predicted values of the logarithmic distribution coeffitients of rT3, T3 and T4 reflect an unambiguous apolar character of the analytes against water. Acetone, acetonitrile, methanol, ethyl acetate seem to be optimal solutes for forced extraction from aqueous matrix, which predictions correlate well with the literature data. Log P values are defined as values of octanol/water log D predicted values.

If predictions reflect the real behaviour of the substances, and so the efficiency and the usefulness of the investigated expert systems, will be presented later in the results and discussion.

3.2. METHIMAZOLE ANALYSIS BY HPLC

3.2.1. COLUMN SELECTION

Before describing method development the main goals of HPLC separation need to be stated:

- the goal of the method development is analysis
- qualitative analysis is needed, since the chemical identity of all sample components is not known
- quantitative analysis is needed in the estimated range of 1 to 30 µg/ml
- method is needed for analysis a large number of samples
- detection should be carry out by commercial detection system (DAD).

Samples for analysis are water and fish whole bodies. Both matrices are soluble: water samples are ready to injection, fish samples contain interferences or column killers that must be removed prior to injection.

Since, no analytical method was known before, we present now the method developing process in details, with presenting all possible effect, which can affect on the separation efficiency.

The main and even the first step of the method development is the right choice of the stationary phase and an appropriate mobile phase to that. At the column selection factors like particle type, particle and pore size, column length and width must be taken into consideration. The using of regular particles or continuously bed (monolithic) column materials is another question to decide before. Monolithic columns do not count to the suitable materials for reproductive measurements not even nowadays. In addition they are not available in wide range on the market or only for higher price. This and the poor reproduction values of analytical results indicate that we gave up the idea to use these materials as a stationary phase for the separation process, since an accurate method for a routine analysis with large number of samples was presumed.

Reversed phase columns were applied since the high polar target compounds were dissolved in aqueous environment and in addition the possible other applications of this method like medicinal laboratory measurements prefer biological friendly eluent systems.

We focused on the well known material bases and commercial available reversed phase regular materials. The tested commercial available analytical columns were selected to represent all the latest developments of reversed phase technology for retaining polar or high polar compounds. Column tests were enabled by manufacturers who placed the columns at ours disposal. And at the same time that was the only limitation factor of collecting information from other new columns.

THE ROLE OF THE COLUMN ON THE SEPARATION

Column specifications of interest include the minimum plate number for a given value of k′, maximum peak asymmetry factor, retention reproducibility, typical pressure drop and bonded phase concentration. Small molecules are usually chromatographed on particles that have 7-12 nm pores. Although smaller particles (e.g. 3 µm) permit faster separation and higher efficiency, 5-6 µm particles generally represent a good compromise in terms of convenience, performance and column lifetime, so we used this particle size during testing different columns. Shorter column length allows shorter analysis time and lower column width can help to reach higher sensitivity. The column plate number is the most important characteristic of a column and a well defined measure of the column quality. N defines the ability of the column to produce sharp, narrow peaks and to achieve good resolution for band-pairs with small selectivity-values, *so we always used well controlled original manufacture products.*

CHARACTERISTIC OF THE COLUMN PACKINGS: THE HYDROPHOBIC SUBSTRACTION MODEL

Reversed phase columns and column selectivity can be characterised by five selectivity parameters, which is formed in the following equation (Eq.14) [*Wilson et al.* 2002; *Gilroy et al.* 2004]

$$\log\alpha = \log\left(\frac{k}{k_{eb}}\right) = \eta H - \sigma S^{*} + \beta A + \alpha B + \kappa C \qquad (14)$$

K represents the capacity factor of a given solute, k_{eb} the value of k for a non-polar reference solute on the same column on the same conditions. The remaining selectivity-related symbols represent either empirical, eluent- and temperature–dependent properties of the column. H characterise hydrophobicity, S* the steric resisitance to insertion of bulky solute into the stationary phase, A is the column hydrogen-bond acidity mainly attributable to non-ionised silanols, B is is the column hydrogen-bond basicity (presently hypothetisized to result from sorbed water in the stationary phase), and C is the column cation-exchange activity due to the ionised silanols. Parameters α', β', η', σ' and κ' denote complementary properties of the solute.

Column characteristic were determined by data obtained from column manufacturer (Bischoff) or measured by ourselves (Atlantis dC18) after the solvent optimisation. On the basis of the similarity of the measured and the literature data, we publish here data measured by *Snyder et al, 2004* because of the consistency and better transparency of the measured values and experimental conditions. But we emphasize at this point, that this published values correspond exactly with the values what we experienced. Our measurements were based on the method by *Cruz et al*, *1997*.The characteristic column parameters are shown in the Tanaka hexagonal [*Kimota et al., 1989*] below at the column information. We present them to demonstrate the potential of the diagrams at the point of column selection.

COLUMNS USED IN TEST

- Nucleosil C18 (ec) columns were purchased from Macherey-Nagel. The column dimensions were 250 x 4.0 mm, 5 µm particle size and 100 A pore-diameter. The ec column is like Nucleosil C18 but the phase is none-polar end capped.

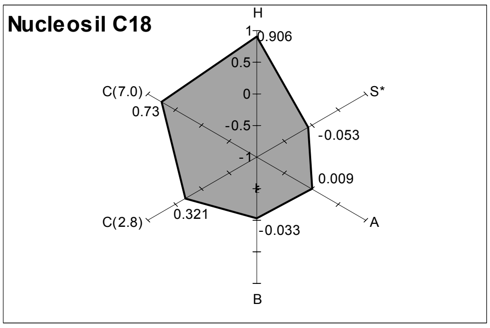


Figure 14. Tanaka diagram of the Nucleosil C18 ec column

- Nucleosil –CN column represents a short chain column (C3-CN) with polar modifying on the end of the alkyl chain (-CN). This column can be used as both normal and reversed phase column depending on the eluent composition and polarity. Advantage of this type of column is the rapid equilibration time. The column dimensions were 250 x 4.0 mm with 5 µm particle size and 100 A pore-diameter, the column was purchased from Macherey-Nagel.

- Prontosil C18 AQ Plus is a phase with similar characteristic to Prontosil C18 AQ, but with more stability even in the lower pH ranges. The 150 x 4.0 mm, 5 µm particle size and 120 A pore-diameter column was made by Bischoff.

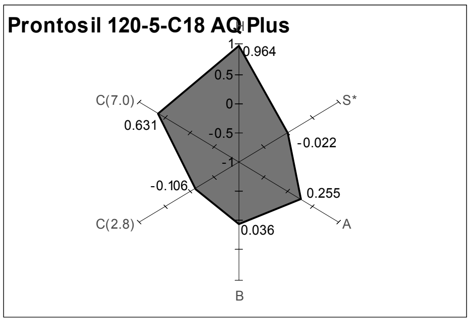


Figure 15. Tanaka diagram of the Prontosil C18 AQ + column

- LiChrospher C18 is an other commercial column with spheric particles and without end capping from an other manufacturer. The125 x x4.0 mm column with 5 µm particle size and 100 A pore-diameter was made by Merck.

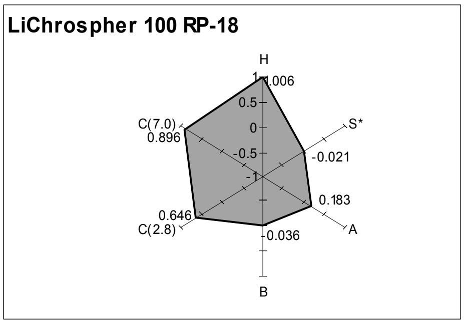


Figure 16. Tanaka diagram of the LiChrospher C18 column

- Discovery HS PEG column was designed for high selectivity and retention polar compounds due to the ether groups of the polyethyleneglykol chains. The 150 x 4.0 mm, *5* µm particle size and 100 A pore-diameter column was purchased from Supelco.

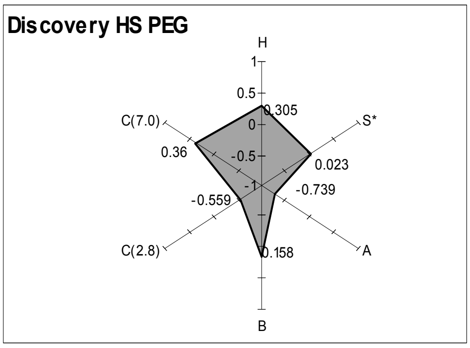


Figure 17. Tanaka diagram of the Discovery HS PEG column

- Multospher RP18 FBS column with a polymer bound reversed phase material on a spheric silica gel particles especially developed for the separation of acids and bases. Due to the RP 18 polymer a high rate of covering of the silica particle is arisen with very high total carbon content. The 125 x 4.0 mm column with 5 µm particle size and 100 A pore-diameter was purchased from CS GmbH.

- XTerra RP 18 is an end capped hybrid particle system material with extended pH stability and extreme homogenous surface covering with 1/3 less surface silanol activity and an embedded polar group direct on the silanol group. The 150 x 4.6 mm 5 µm spheric particle and 125 A pore-diameter 100% water compatible column was made by Waters.

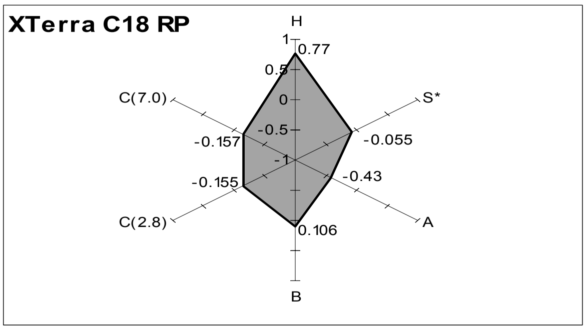


Figure 18. Tanaka diagram of the XTerra C18 RP column

- Atlantis dC18 phase is a difunctionally bounded, partial endcapped reversed phase, which offers a perfect balance of reversed phase retention for polar and non-polar substances. The with 100 % water compatible column with 150 x 4.6 mm length and width, 5 µm spheric particle size and 125 A pore-diameter was purchased from Waters.

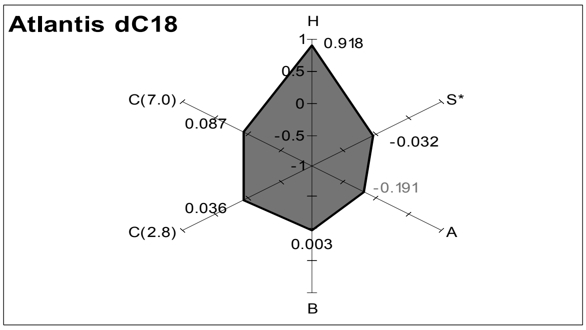


Figure 19. Tanaka diagram of the Atlantis dC18 column

Table 16. Summary of the parameters of the tested columns

Column	*Manufac.*	*L x w [mm]*	*Particle size [µm]*	*Pore size [Å]*	*Carb. Cont. [%]*	*Surface area [m^2/g]*	*basic*	*pH*	*End capping*
Nucleosil C18	Macherey - Nagel	250 x 4.0	5	120	11		Silica	2-8	Non E.C.
Nucleosil C18 EC	Macherey - Nagel	250 x 4.0	5	100	11		silica	2-8	E.C.
Nucleosil-CN	Macherey - Nagel	250 x 4.0	5	100	n.A.		Silica	2-8	Non E.C.
ProntoSil C18 AQ Plus	Bischoff	125 x 4.0	5	120	17	300	Silica	2-8	E.C. base-desaktivated
LiChrospher C18	Merck	125 x 4.0	5	100	21		Silica	2-8	Non E.C.
Discovery HS PEG	Supelco	125 x 4.0	5	120	12	300	Spheric Silica	2-8	E.C.
Multospher RP 18 FBS	CS	125 x 4.0	5	100	25		Silica	2-8	
XTerra RP18	Waters	150 x 4.6	5	125	15	175	Spheric Silica	1-12	E.C.
Atlantis dC18	Waters	150 x 4.6	5	100	12	330	Spheric Silica	2-8	E.C.

3.2.2. ELUENT AND COLUMN TYPE OPTIMISATION

DETERMINATION OF THE OPTIMUM SOLVENT STRENGTH

Conditions that produce the preferred solvent strength are best determined empirically. One approach is to begin with a mobile phase that is probably tot strong and reduce strength to increase retention between successive runs. When all bands fit within the range of $1<k'<20$ the mobile phase is near optimum from the standpoint of solvent strength. Intermediate values of k' are desirable in the range of $1<k'<20$ for all peaks in the chromatogram. Even when the separation of peaks with $k'<1$ is possible, the resulting separation will usually be unsatisfactory: interfering bands are more likely in the region $0<k'<1$ and poor baselines that make quantification difficult often are observed in this part of the chromatogram. For this reason, every effort should be made to avoid conditions that lead to peaks with $k'<1$.

Once the solvent strength has been properly adjusted for the sample, the next separation parameter that can be explored is selectivity (α). In many cases, it is possible to obtain a satisfactory separation simply varying solvent strength.

Mobile phase composition has a major effect on peak spacing: no other variables will generally prove as powerful for controlling values of selectivity.

In the first step of the method optimisation weak eluent strength solvents: 100% MeOH and 50 % MeOH/water solutions were performed as eluent. Capacity factors of standard reference solutions were observed versus organic content of the eluent. Standard solutions were injected in three parallel measurements in all case of all columns. All of in the following presented measurements were carried out with 10 ppm concentration standard mixed samples. Detection wavelengths were 255 and 220 nm. The 50% and 100% MeOH results are shown in the following figures (Fig. 20-25).

The mark MSO represents the two co-eluated sulfoxidised (MSOH and MSO2H) metabolites.

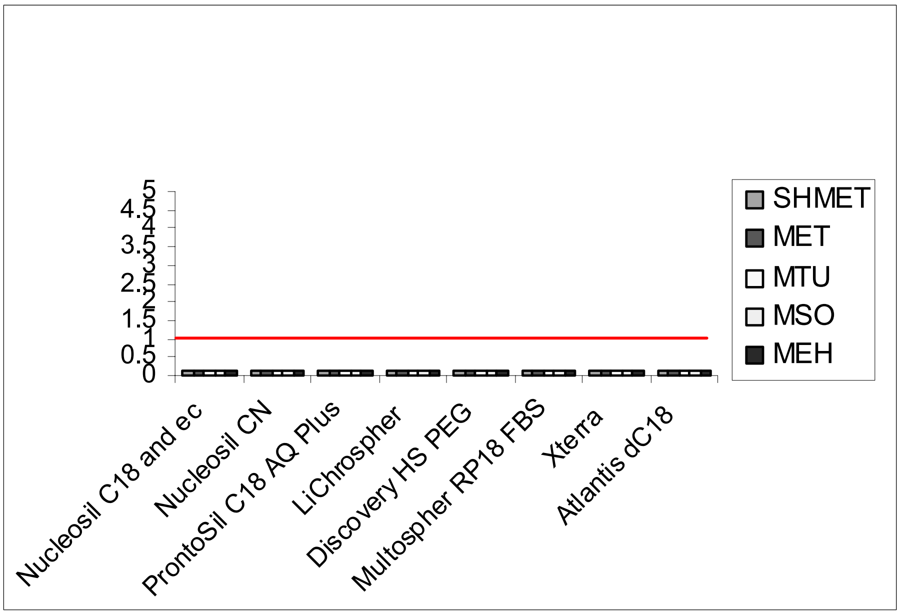


Figure 20. Measured capacity factors of the analytes on the different tested columns. Samples were eluted with 100% MeOH. Red line represents the required minimum capacity factor value.

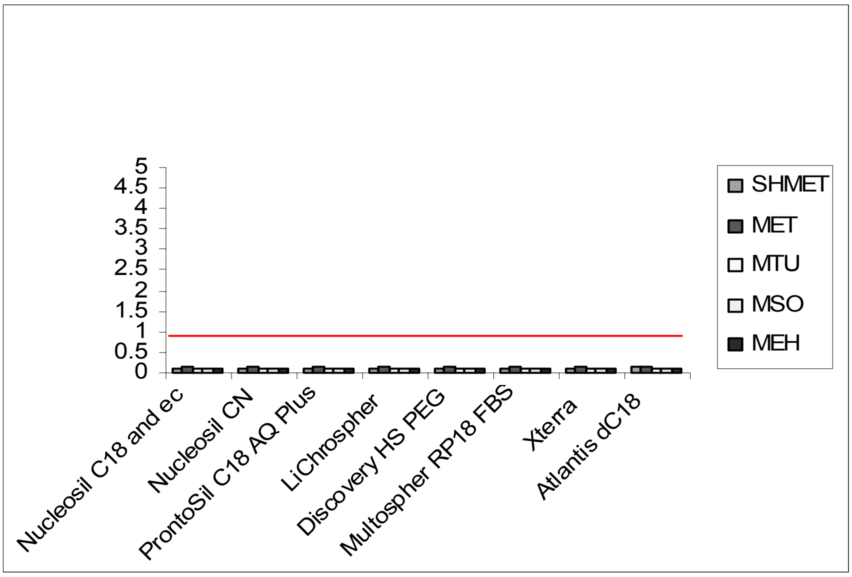


Figure 21. Measured capacity factors of the analytes on the different tested columns. Samples were eluted with 50% MeOH/H_2O. Red line represents the required minimum capacity factor value.

Relative capacity factors were determined between 0.10 and 0.15 with the weakest organic solvent MeOH. Acetonitrile as another possible organic solvent that is simultaneously stronger solvent than methanol resulted more less capacity factors: values between 0.11 and 0.13. Other commercially used organic solvents in reversed phase chromatography like chloroform, dichloromethane, esthers or ketones were not suitable for application because of the limited solubility of the target compounds in them. Since intermediate values of k´ are desirable in the range of 1<k´<20 for all peaks in the chromatogram, values under k=1 are not accepted and so the organic content of the eluent must have been reduced. For further experiments Methanol was as organic solvent applied, acetonitrile was avoided due its stronger feature. Measured capacity factors with weaker eluent compositions are discussed in the followings. The effect of 10 percent methanol in the eluent is shown in Fig. 22.

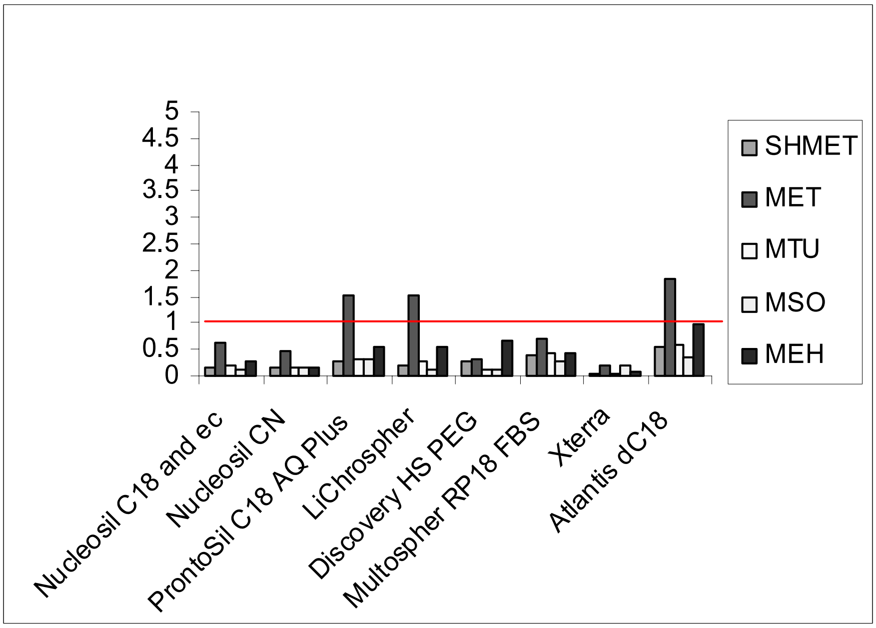


Figure 22. Measured capacity factors of the analytes on the different tested columns. Samples were eluted with 10% MeOH /H_2O. Red line represents the required minimum capacity factor value.

10 percent of organic modifier in the eluent resulted higher retention in all case of columns and substances than higher organic content of the mobile phase. Although only one compounds of interest (Methimazole) were retained satisfactory. The measured retention values fulfilled the criteria k′>1 but only in case of ProntoSil C18 AQ Plus, LiChrospher RP 18 and Atlantis dC18 columns. Further optimisation steps were needed to achieve the chromatographic criteria simultaneously for all components of the interest. The organic content of the mobile phase were reduced in the following steps. 5% methanol-water and 100% aqueous eluent were applied. The results are seen in the Fig. 23.

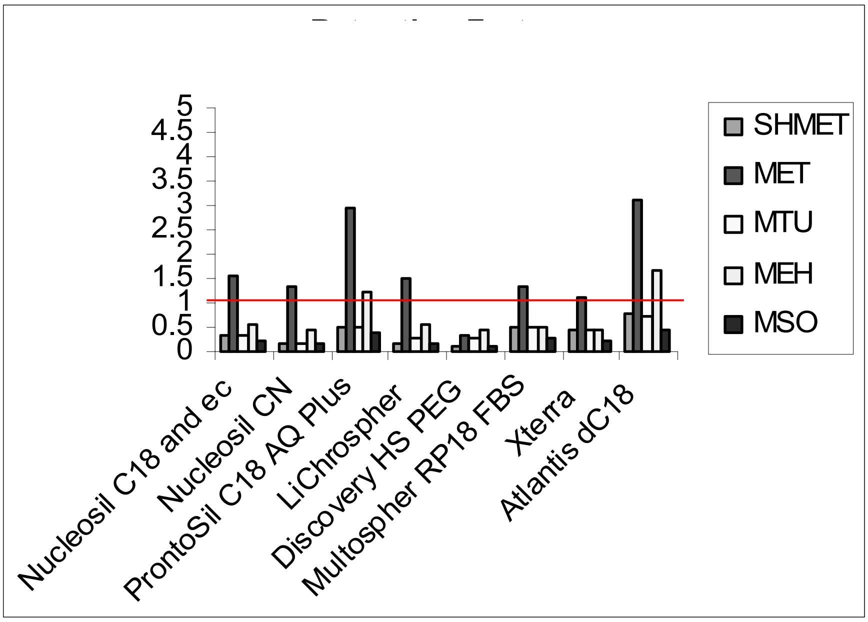


Figure 23. Measured capacity factors of the analytes on the different tested columns. Samples were eluted with 5% MeOH/H_2O. Red line represents the required minimum capacity factor value.

Decreasing of the organic rate in the mobile phase (decreased eluent strength) resulted higher retention valuesin all case of columns. Methimazole was satisfactory retained with all of the tested columns except the Discovery HS PEG. The oxidized metabolite N-methylhydantoine got connected to the group of well retained compounds with k′>1 in the case of the ProntoSil and the Atlantis columns. No other tested substances were retained in the solid phase fulfilled the chromatographic criteria with neither of the tested columns. 100 percent water as weakest mobile phase was the final option to achieve a proper chromatographic method in the reversed phase modus. The results are shown in the Fig.24.

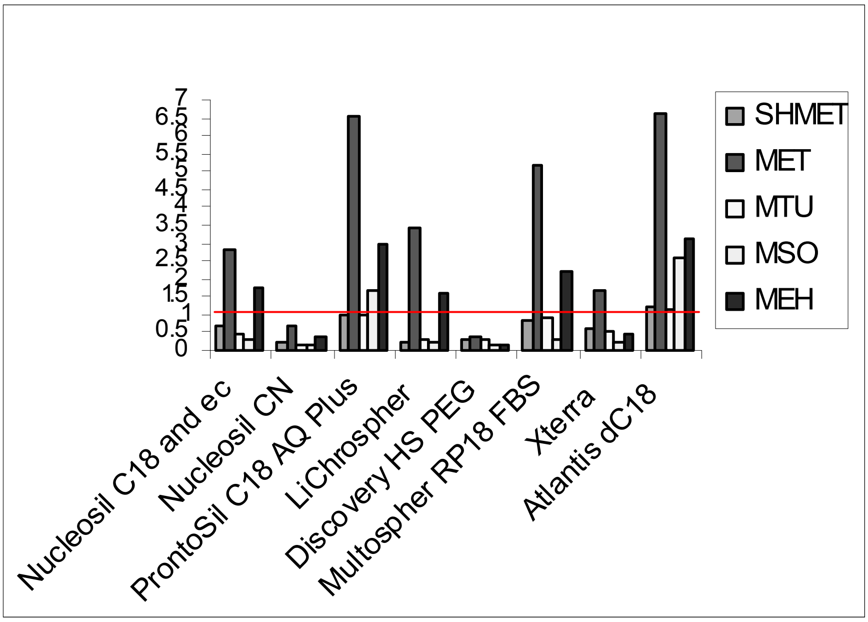
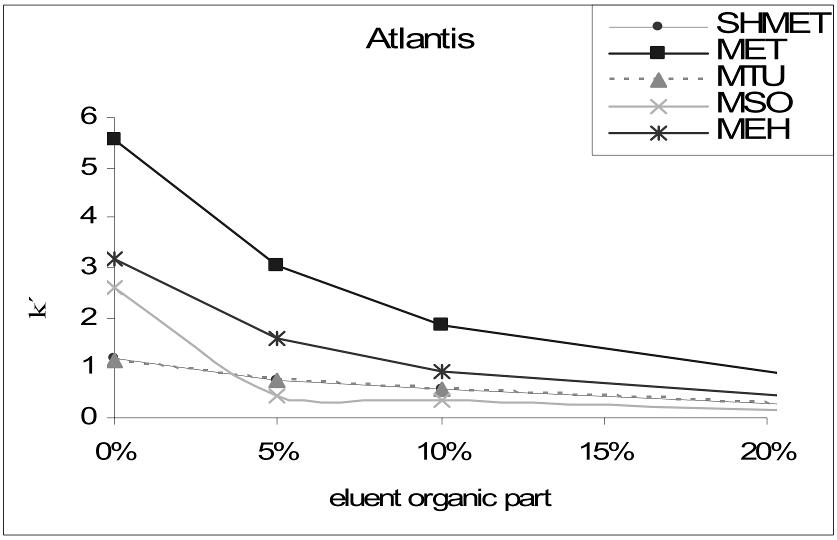


Figure 24. Measured capacity factors of the analytes on the different tested columns. Samples were eluted with the weakest eluent, 100% H_2O. Red line represents the required minimum capacity factor value.

As expected the weakest eluent strength solvent in resulted the highest capacity factors for all compounds. The majority of tested columns were unable to retain compounds meet with the requirements. Only two of tested columns: ProntoSil C18 AQ Plus and Atlantis dC18 (in the following Prontosil and Atlantis) were suitable for satisfactory retention of methimazole and its metabolites simultaneously within a single run. The measured k´ values were 1.20 for SHMET, 5.52 for MET, 1.15 for MTU, 2.59 for MSO and 3.13 for MEH, respectively in the case of the Atlantis column. Capacity factors with Prontosil column were 0.99 for SHMET, 6.58 for MET, 0.99 for MTU, 1.67 for MSO and 2.99 for MEH, respectively. k´ values as 0.99 (for SHMET and MTU) measured by Prontosil column were considered equivalent with the critical requirements (k´=1).

Neither of k´ values exceeded the maximal accepted range k´>20.

Measured capacity factors as function of the organic content of the eluent are pointed out in the Fig. 25 in the case of the two suitable columns for further method optimisation.



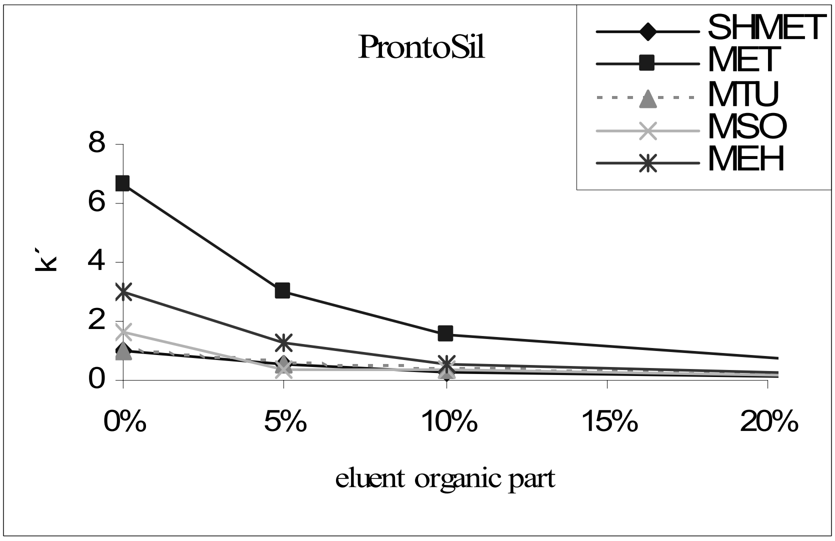


Figure 25. Measured capacity factors in function of the organic content of the eluent obtained with the Atlantis and the Prontosil columns

Peak shapes, peak symmetries were proper. Resolution was found not suitable neither in the case of MTU/SHMET (R_s=0.09 for Atlantis and R_s=0.11 for Prontosil) nor MSO, but appropriate (R_s>>1.5) for the other substances. MTU, SHMET and MSO showed minimum capacity factor in the presence of 4% methanol in the eluent.

The effect of the eluent organic content on the measured theoretical plate numbers and so on the peak broadening and separation efficiency is represented in the figure 26.

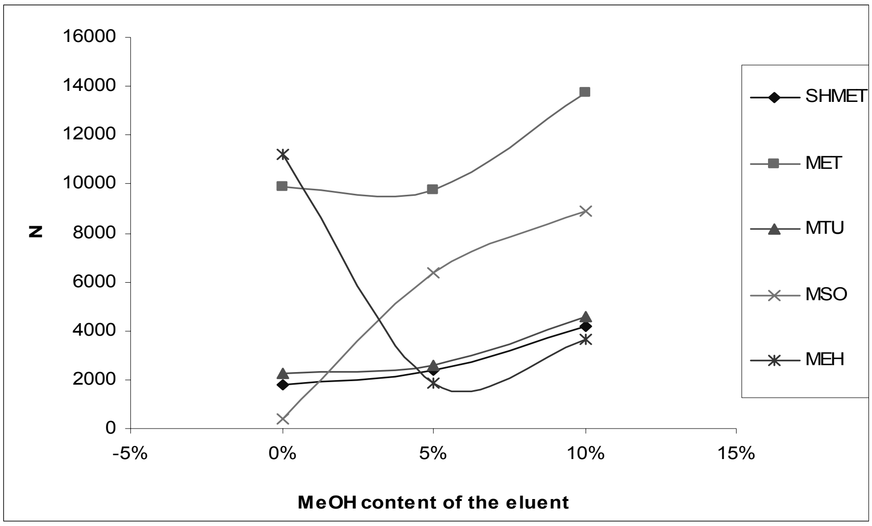


Figure 26. Effect of the eluent organic content on the theoretical plate numbers in the case of the Atlantis column

It is shown from the plots, that theoretical plate numbers were increasing with higher methanol content in the eluent. Opposite to these expectations, MEH showed decrease in the H number at higher organic content. Higher interaction of the surface silanols with the MEH could be conceived as reason for that effect.

It can be concluded at this point that:

- there was found two columns suitable for retaining all of the target analytes fulfilled the basic and most fundamental criteria: k′>1
- probably 100% or high aqueous solvent ratio in the mobile phase is needed for the separation as initial
- four of six compounds can be separate with mobile phase optimisation
- further optimisation steps are necessary to improve separation. The co-eluted compounds MTU/SHMET and the two sulph-oxidized metabolites (MSO) must be optimized to achieve a more powerful separation
- effect of other separation variables has to be examined and optimised.

3.2.3. OPTIMISATION OTHER SEPARATION VARIABLES

When two or more peaks are still unresolved after solvent optimisation, there are other possibilities to improve the separation efficiency.

These are in principle:

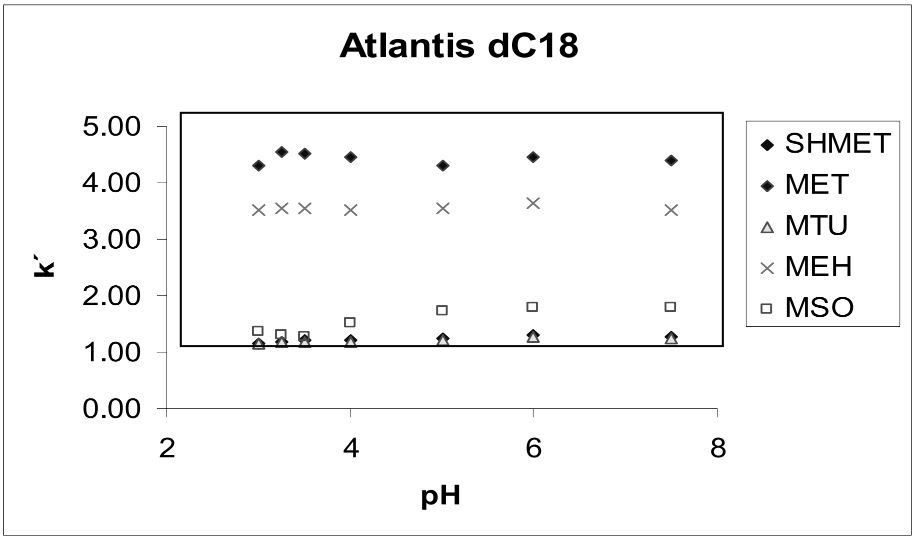
- changing of ionic strength (buffer concentration)
- changing of pH of the mobile phase
- choosing an other column type
- using mobile phase additives
- purchasing the same column from different source
- temperature changing
- additional organic solvents.

As it was shown above, various different column types were tested and only two of them were found suitable. We passed on adding other organic solvents to the mobile phase, thus not even the weakest solvents resulted proper retention in some cases. Changing pH, temperature and ionic strength of the eluent were established.

3.2.4. BUFFERS AND PH

If samples contain acidic or basic compounds, the retention can vary with both ionic strength and pH. An increase in ionic strength reduces the retention of protonated bases on column packings with ionised, residual silanols. Sufficiently high salt concentration can increase the retention of neutral compounds.

The whole working range of the columns (pH 3-7.5) was tested from the viewpoint of retention behaviour of the compounds. 10 ppm standard mixed samples were injected. Samples were eluted with formiate or phosphate solution in the pH range 3-5 and with phosphate (0.05 M) in the range pH 5-7.5. Capacity factors against pH curves are shown in figure 27.



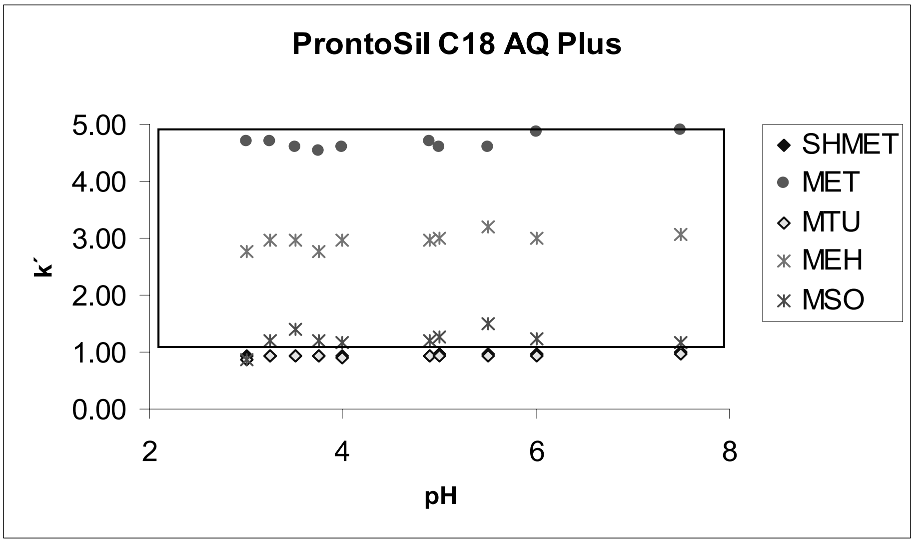


Figure 27. Capacity factors in the function of the eluent pH in the case of the Prontosil and Atlantis columns. The pH was adjusted with phosphate.

As above figures show the proper retention and resolution values of MET, SHMET, MTU and MEH were not influenced by changing pH value of the mobile phase. The observed fluctuation of the measured k´ values at different pHs were within the relative standard deviations of the precision (see later in part Validation of the method) for all analytes. We have to emphasize the importance of the phenomena could be observed on the MSO course.

MSO showed sufficient retention during organic solvent optimisation, which can also be obtained in the whole pH range 3-7.5. Below pH 6 significant decreasing of the retention were seen in case of Atlantis column. Observed capacity factors of MSO came near to the critical value k′=1 in the lower pH range (3-3.5) measured with the Atlantis column. In the case of Prontosil column this phenomena could be observed only at lower pH levels (pH ~3). This phenomenon could be explained as result of the suppressed dissociation of the sulphanyl and sulphynil groups due to the low pH. Due to the associated (hydrogenated) –SOH and –SO_2H functional groups, increased hydrophobicity thus increased retention could be detected. The other tested substances appeared not to be ionisable in the column pH-working range. From the viewpoint of the separation it does not mean any difficulty, since hereby the robustness of the already known good separation was verified. As it is seen in the figures, no other optimisation step is necessary to separate MSO from the other components fulfilled the k′ criteria. MTU and SHMET are still not to be separated none of the pH values.

Since Xterra column has the widest pH-range among of the tested columns, we used it for testing effect of extreme pH value on the retention. However, the k′ values were not satisfactory on the column not even with applying the weakest mobile phase, enormous changes in the chromatographic behaviour of the substances (e.g. unexpected (de-) protonisation of the analytes or silanol groups of the column) could have been noted by observing e.g. unexpected increased or decreased retention times or changing in peak forms or shapes. Standard samples were injected and eluted with 100% pH 7 Millipore Milli-Q water and pH 10 glycine buffers. The relative extreme pH value had no effect on the retention parameters, thus no protonisation-deprotonisation effects were observed. Peak shapes and symmetries were as before, so no effect was observed by using high pH (Fig. 28.). Observations pointed out, that the pH-robustness of the separation using standard samples was excellent.

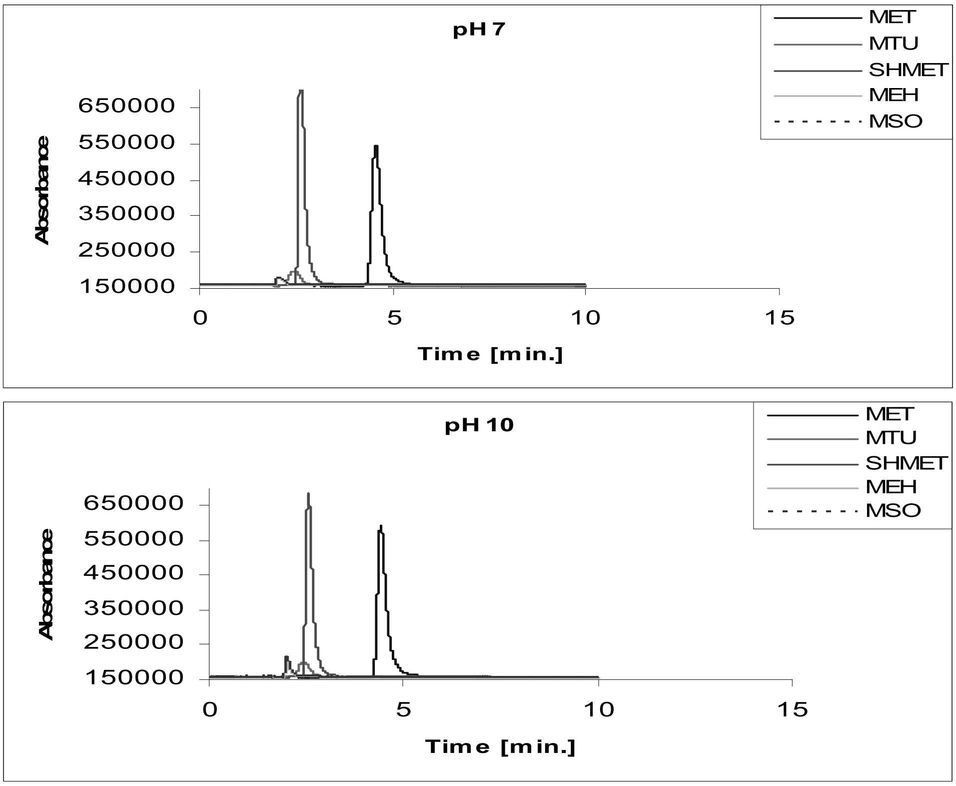


Figure 28. Chromatogram outfits obtained by the XTerra column at extremely high pH value (pH10) compared to pH 7

3.2.5. THE ROLE OF THE BUFFER QUALITY

In working ranges of the columns is the usage of different buffers possible. The effect of the buffer quality on the retention was checked in the range pH 3-4 with both columns Atlantis and Prontosil. Results with phosphate were presented above, values using formic acid are presented in Fig.29.

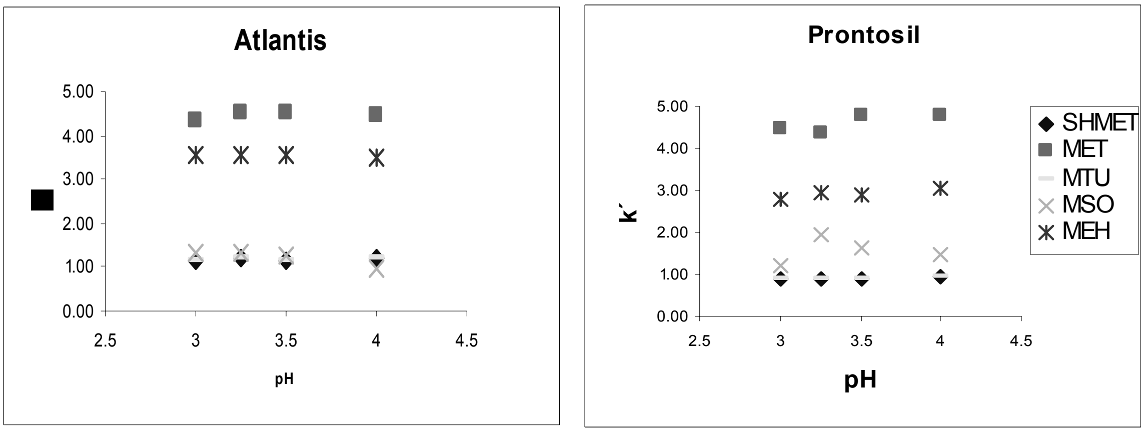


Figure 29. Capacity factors depending on the buffer quality by using formic acid to adjust pH. Tested columns were Atlantis and ProntoSil.

Capacity factor values of SHMET, MTU, MET and MEH were similar to the results obtained by phosphate. However, runs with phosphate resulted more accurate points with both formiate and phosphate, using phosphate on the Prontosil column showed higher scattering on the plot, than in the case of formiate.

Resolutions of peaks MTU/SHMET and the sulph-oxidized compounds (MSO) were not higher than with phosphate. Poor resolution of the peaks MSO and SHMET/MTU could be observed in the case of Atlantis column in the whole tested range and in the case of Prontosil column at the lowest tested pHs. Sigbificant increasing of the retention was noted between pH 3.3 and 4 with formic acid on the Prontosil column. Increased silanol group ionisation (pH is about the expected pKa value of the column) and followed formation of more and stronger interaction between –SOH, -SO2H groups of the analyte and the stationary phase silanols can be hypothesised as reason for that behaviour.

Important practical observation was the increased amount of ghost peak formation and unambiguous noisier baseline in the case of using formic acid or ammonium-formiate salt at the detection wavelength 220 nm with both columns. The usage of acetate as buffer component was found contraindicated: too noisy baseline and following lower minimum amount of detectable mass was observed.

3.2.6. EFFECT OF BUFFER CONCENTRATION

As it was discussed before, changing in ionic strength can affect on the separation performance. Effect of change ionic strength due to change buffer (salt) concentration was examined with diphosphate salt and phosphate buffer in the pH range between 3 and 7.5.The pH values lower than 4.5 were adjusted by adding of the appropriate amount of 50% phosphoric acid to the diphosphate solution with given salt concentration. Effect of buffer concentration was determined in the range 0-100 mM. Buffer concentration if they were present at least in 50 mM concentration showed no changes on the chromatographic parameters in the case of the studied substances with exception of MSO. MSO showed high sensitivity to buffer presence in the solution. With using Milli-Q quality water (pH 6.8-7.5) unshaped tailing peaks and raised retention time was observed with the Atlantis column. Similar unshaped and tailing peaks but no considerable retention time changes was seen using water as mobile phase with the Prontosil column. Really shape, symmetric peak forms were formed by adding inorganic salts to the aqueous mobile phase adjusted to the same pH.

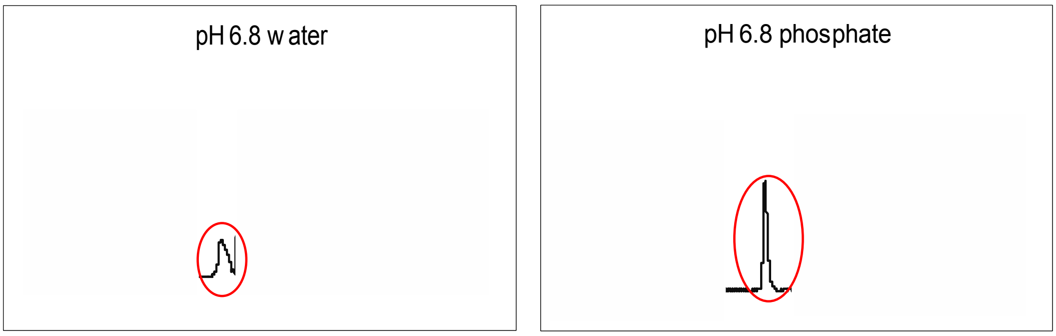


Figure 30. Effect of the buffer concentration on the peak shapes. MSO peak showed high sensitivity to the presence of buffer salts: peak got sharper and more symmetrical.

3.2.7. MOBILE PHASE ADDITIVES

Compounds that can interact with the silanols of the column packing can be added to the mobile phase to control band spacing, reduce peak tailing and peak broadening. Cationic samples will normally show decreased retention. However, there was no observed any abnormalities on the standard peaks, that could not been eliminated by changing one of the separation variables during the previous optimisation steps, we checked the effect of triethyl-amine and ion pair reagent pentanesulfonic acid sodium salt on the separation for the sake of order.

Effect of triethyl-amine on the separation performance is introduced in the figure 31.

The observed drastically baseline drifting did not affect the metabolite peaks but influenced the baseline flat and the signal to noise ratio (and following the minimal detectable substance mass also) in the time window where among others the mother substance will be eluted. This and the reason that the detected peak forms do not demand the usage any additives in the mobile phase we do not propose their application to the eluent.

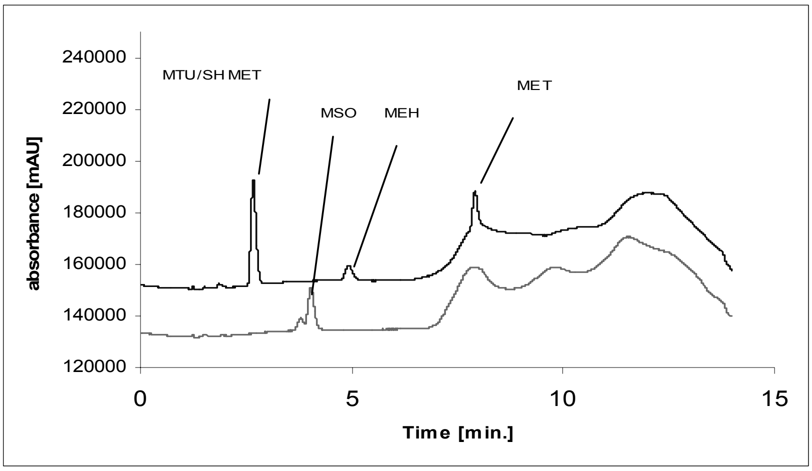


Figure 31. Effect of triethylamine presence in the mobile phase on the baseline noise. Explicit baseline drifting was obtained in the range of the methimazole retention time.

Effect of C_5-ion-pairing reagent was also investigated, and decreased but no significant change in the retention was detected with substances SHMET, MTU, MEH and MET. Significant decreasing in retention time was to obtain in the case of MSO. The retention time showed 40% decreasing in retention time at pH 3.65 with the Polaris column and 11% with the Atlantis column at the same pH without any essential changes in other peak parameters.

3.2.8. EFFECT OF TEMPERATURE

Normally the temperature has only a minor effect on the band spacing. However it can be important if two peaks are still unresolved after changing the major separation variables and therefore is occasionally used as a variable for controlling band spacing. However, when solvent strength is adjusted to compensate for decreased retention at higher temperatures, the effect on selectivity is often small. Since the column plate number usually is larger at higher temperatures, the maximum resolution will occur at higher temperature regardless of bands spacing [*Snyder and Dolan,* 2000*; Zhu et al.,* 1996].

Temperature effects were tested with Atlantis column applied the optimized mobile phase (s. details below). The normal temperature range of the column was defined between 15-40°C according to the manufacturer's guide. The values 20°C, 30°C and 40°C were set.

The measured retention times and the formation of the theoretical plate number values are show in the Fig. 32.

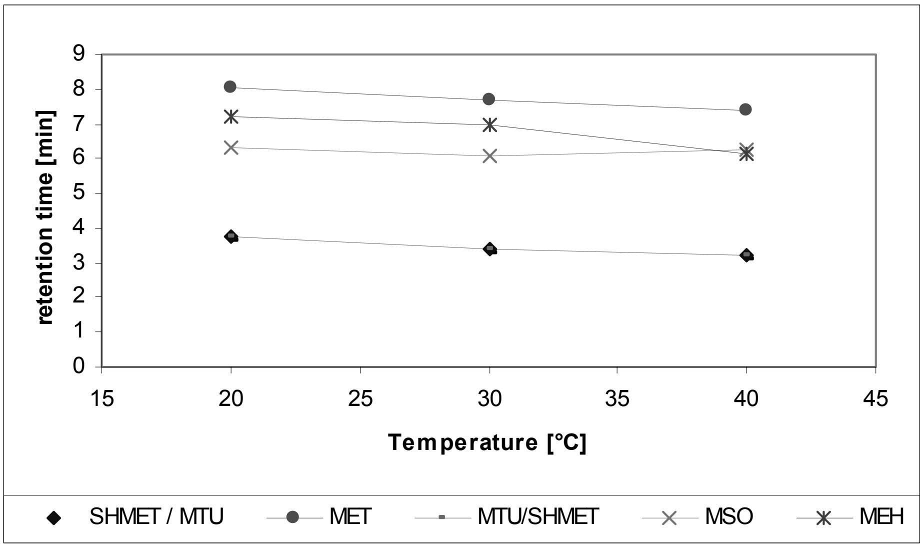


Figure 32. Retention time as function of the temperature on the Atlantis column

Retention times of SHMET, MTU and MEH were decreased by 15 % and MET by 8%. MSO showed 8 % lower retention at 30°C than did at 20°C and than 8% higher at 40 °C than at 30°C. The increased MSO and the decreased MEH retention times caused lower selectivity of both peaks and led to a total loss of resolution at 40 °C (Table 17).

Table 17. Changes in the resolution factor of MEH and MSO at different column temperature

Temperature [°C]	Resolution MEH/MSO
20	2.78
30	2.56
40	0.15

The effect of temperature on the theoretical plate number is presented in the Fig.33.

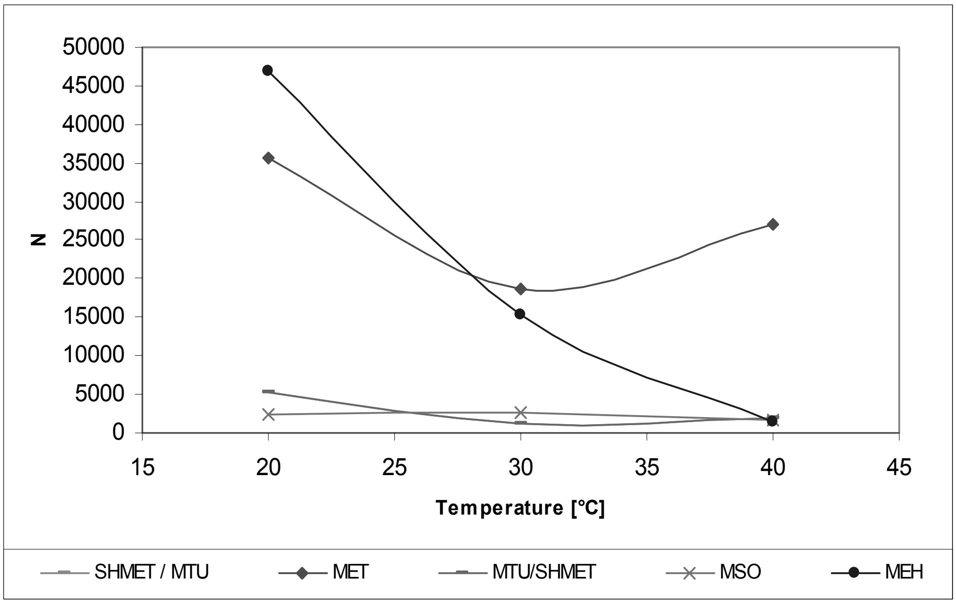


Figure 33. Effect of the temperature on the theoretical plate numbers. Determination was carried out with the Atlantis column.

As it was mentioned above higher temperature usually causes smaller peaks and so higher plate numbers. Our measurements with the Atlantis column did not verify this observation with our substances. MEH showed an unambiguous peak broadening measured on the baseline, and so more than 90% loss on the plate number. Methimazole baseline width became 48% greater at 30 °C and again lower at 40°C (N=27078), but not as high as it was at 20°C (N=35580). Here must be noted, that the measured high theoretical values (more than 10.000-12.000) are only apparent values: the outcome of using gradient elution.

Similar trends were observed on the course of the substances MTU and SHMET. Plate number N=5147 at 20°C descended to 1315 at 30°C and then climbed again up to N=1815 at 40°C. MSO showed other characteristic: after a value of N=2316 at 20°C, maximal plate number was achieved with N=2737 at 30°C, and a minimum value N=1779 was measured at 40°C.

It can be concluded, that lower column temperature is preferred for a method with good performance. Further measurements were carried out at ambient temperature.

3.2.9. METHOD OPTIMISATION: DESIGN OF THE GRADIENT ELUTION

Changing in resolution, decreasing capacity factors or time saving are the reasons for the usage of gradient elution. However, the previous measurements provides that a higher resolution of problematic peaks could not to be achieved by using gradient elution and in fact there is no need for drastically decreasing the retention times of the late eluted substances, we built up a gradient method in order to save time during the routine sample measurement. The gradient elution was carried out with pH 5 0.05 M phosphate buffer and methanol as strong eluent. During the gradient developing the values of the theoretical plate number, the resolution and the retention just as column equilibration were optimised. In gradient elution, it is important that the column be completely equilibrated with the strong mobile phase before injection the next sample and the beginning the gradient. Equilibration usually can be achieved by flushing the column with 15-20 column volumes of the starting mobile phase. Symptom of an inadequate column equilibration is a variation in retention time for early peaks in the chromatogram that is in the case of our high polar substances not acceptable. However, the recommended flushing volume and thus the equilibration time is very high, there are possibilities to reduce this additional time: e.g. flow rate during column regeneration can be increased. We used an autosampler for injection, so that the time between injections was kept constant. In this case, (if) the column was not completely equilibrated at the time of sample injection, but the column was in a constant, semi equilibrated state for each sample.

The applied relatively short equilibration time after the gradient run and the initial step before injection was to be found enough for the column regeneration. However, the calculated optimal gradient time (t_g) was 3.12 min. , we decided to run the gradient program longer (6 min.) to be able to remove more effective the contaminations retaining on the column deriving from a complex biological matrix as was fish whole body in our case.

The optimised gradient parameters for the separation are presented in table 18.

Table 18. The optimal gradient programming for the separation of methimazole and its metabolites

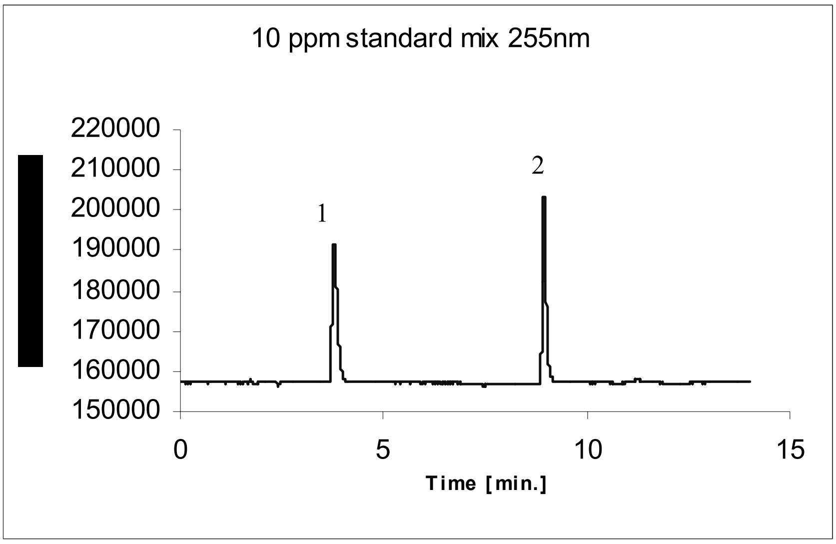
Time [min.]	% buffer	% methanol	Gradient grade
-1	100	0	
0	100	0	
2	100	0	
4	90	10	1
8	90	10	
9	100	0	1
14	100	0	

3.2.10. VALIDATION OF THE METHOD

The validation of the optimised chromatographic method was performed according to the principles detailed in the chapter 2.9.

PEAK IDENTIFICATION (QUALIFICAION)

Typical chromatograms of an injected standard sample (10 ppm µg/ml) measured with the optimised method are presented in the figure 34.



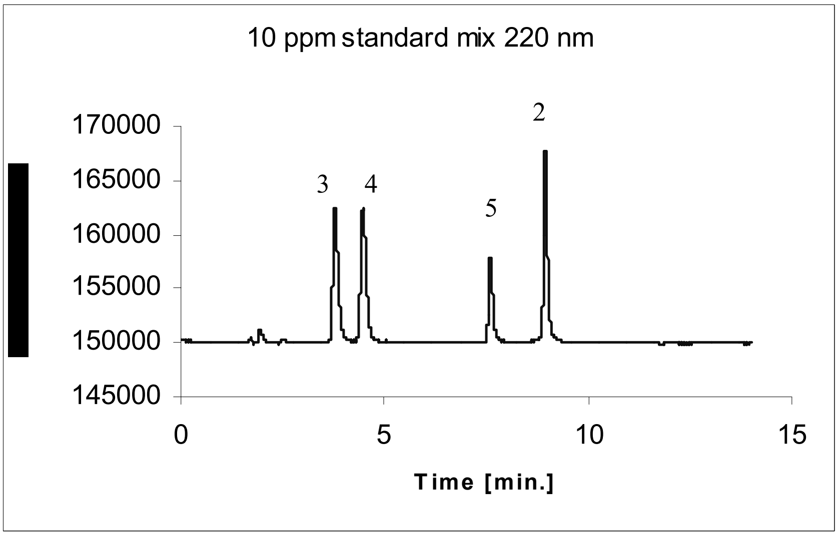


Figure 34. Typical chromatograms of a 10 ppm standard sample detected at 255 nm (above) and 220 nm (below). Elution order was: 1:SHMET, 2:MET, 3: MTU, 4: MSO, 5: MEH.

Good performance of the separation could be obtained on the chromatograms measured by the optimised method, although a satisfactory separation of both sulph-oxidised and MTU/SHMET compounds could not be achieved. Five of six compounds of interest (MET, SHMET, MTU, MEH and MSO) were to be identified by the method; the single (MSOH) and double (MSO_2H) S-oxidised metabolites of methimazole were neither separable nor identifiable: they were eluted as one peak.

In case of all peaks the values of retention time, capacity factor, resolution, peak symmetry, theoretical plate number were measured. Relative standard deviation values of the retention times were determined both between and in-between days.

Table 19. Qualification parameters of the HPLC method for the methimazole analysis. (Values with * are apparent values.)

	t_r	k′	Precision of the qualification		As	N
			%RSD t_r intra day	*%RSD t_r inter day*		
SHMET	3.78	1.29	1.4	2.1	1.13	3381
MET	8.67	4.25	2.3	6.8	1.12	41616*
MTU	3.7	1.24	0.7	1.6	1.2	4525
MSO	4.39	1.66	2.5	2.7	1.08	9517
MEH	7.53	3.56	1.3	3.6	1	90721*

Due to the gradient optimisation all capacity factors were found within the required retention range 1<k′<10. All symmetry factors were in the recommended range 0.9<As<1.2, the relative standard deviation values showed high precision of the method. Theoretical plate values back up a satisfactory low peak broadening value. The unusually high N values of MET and MEH are apparent N values: they are higher in consequence of the gradient run. Resolution values were determined for all substances in all relation (Table 20).

Table 20. Resolution factors of the optimised HPLC method for the methimazole analysis

	SHMET	MET	MTU	MSO	MEH
SHMET	X	X	X	X	X
MET	15.77	X	X	X	X
MTU	0.21	16.10	X	X	X
MSO	1.69	14.50	1.89	X	X
MEH	13.41	5.42	13.60	11.82	X

The measured R_S>1.5 values correspond to the requirements. Resolution of the S-oxidised metabolites equals to zero so thus no identification of the metabolites is possible. On the basis of this phenomenon we discuss these two compounds as one in the following. MTU and SHMET showed no better resolution (R_S=0.21) after optimisation of the separation variations, but they were to identify and quantify due to their different absorbance characteristic (s. below).

QUANTIFICATION

Qualification and quantification were carried out at different wavelength: MTU, MSO and MEH were detected at 220 nm, while SHMET and MET were detected at 255 nm. However, the peaks were overlapped, the different molecular absorption coefficients made it possible to quantify the not separated SHMET and MTU by detection at two parallel wavelengths. Effect of the detected peak area ratios on the detection error is shown in the figure 35.

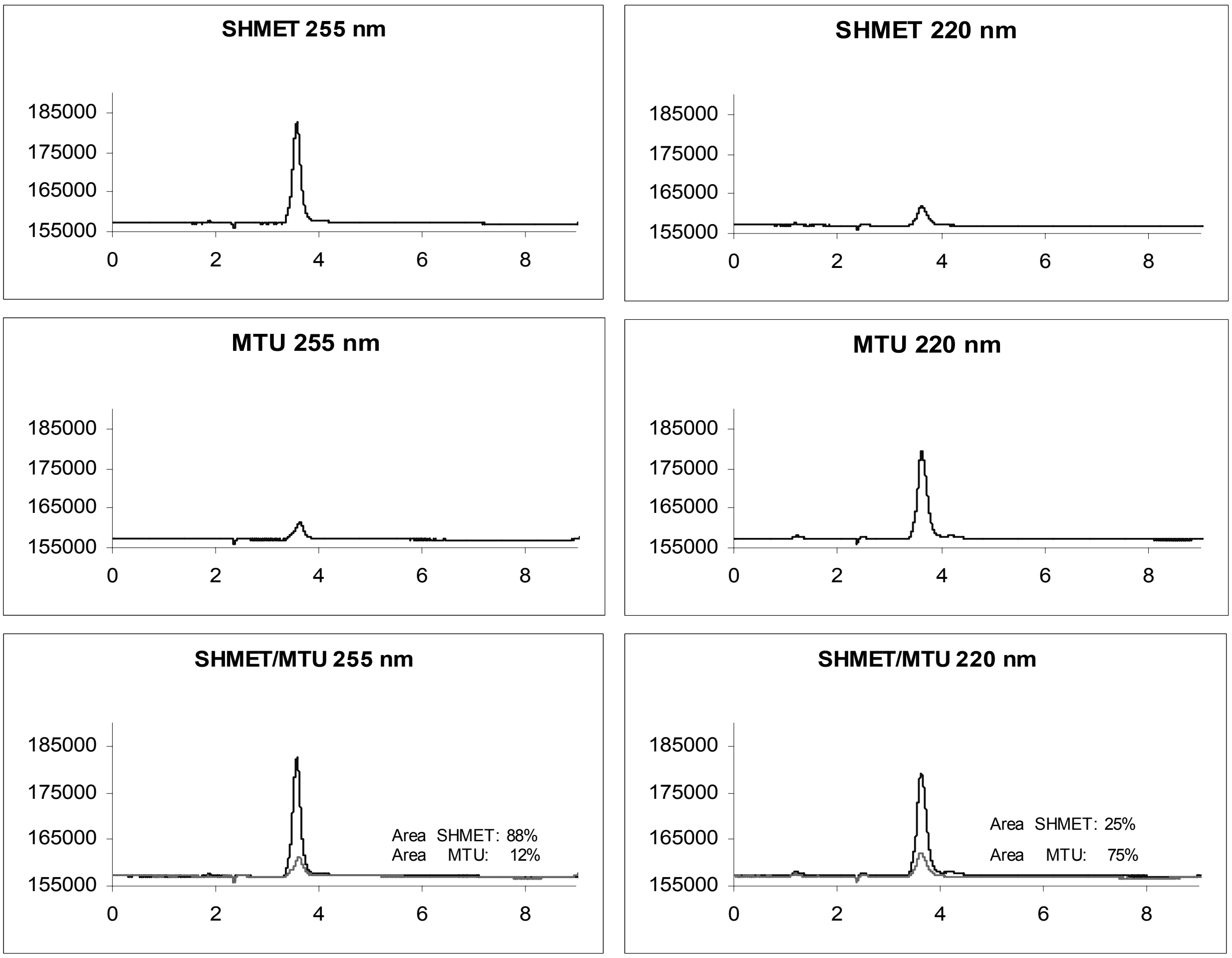


Figure 35. The error of the quantification of MTU and SHMET determined by the peak area ratios at 255 nm and 220 nm

Peak area ratio of the SHMET in the overlapped SHMET/MTU peak at 255 nm was given as 88% of the total measured peak area. 12% of the total peak area was given as the MTU ratio. At the detection wavelength 220 nm higher peak height and/or peak area overlapping was to observe: 75% of the total peak area derived from the MTU and 25% from the SHMET. In the spite of the non-satisfactory resolution of the compounds we accepted the application of these overlapped peak area values for the quantification and the evaluation process of SHMET and MTU. This error in quantification was reduced by taking these measured values with correction by the determined overlapping peak area ratios into consideration. The error of qualification was reducible with recording and evaluating the UV spectrum.

CALIBRATION

300 µg/ml MET, SHMET, MEH, MTU and MSO stock solutions were prepared in water and later mixed and diluted in eight different concentrations for external calibration. The concentration of standard solutions ranged from 0.1 to 100 µg/ml. Calibration points were 0.1, 0.5, 1, 3, 5, 10, 30 and 100 µg/ml concentrations. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration. The standard calibration curves of peak area against concentration of MET, SHMET, MTU, MEH and MSO are shown in the figure 36.

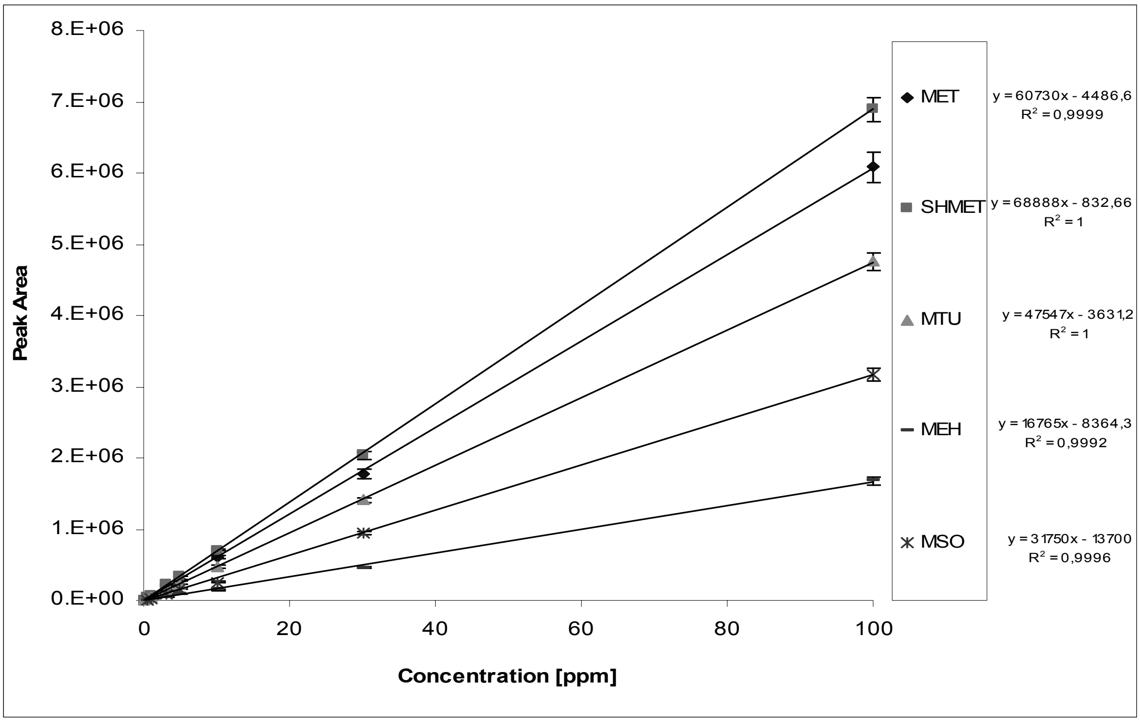


Figure 36. Calibration curves of the methimazole and its metabolites

The measured slope, intercept, correlation coefficient of each calibration curves and the calculated LOD and LOQ as well as the precision are shown in table 21.

Table 21. Quantification parameters of the methimazole analysis.

Component	Calibr. range [ppm]	Slope	Intercept	Corr. coeff.	Sensitivity		Precision of the quantfication	
					LOD	LOQ	%RSD peak area intra-day	%RSD peak area inter-day
SHMET	0.1-100	68888	-832	1	0.06	0.13	2.4	6.3
MET	0.1-100	60730	-4486	0.9999	0.06	0.13	3.6	7.9
MTU	0.1-100	47547	-3631	1	0.10	0.20	2.6	8.5
MSO	0.1-100	31750	-13700	0.9996	0.25	0.50	2.7	7.7
MEH	0.4-100	16765	-8364	0.9992	0.43	0.86	3.0	9.6

The linearity of standard calibration curves was confirmed by the high values of the correlation coefficients. Limits of detection and limits of quantisation were determined or quantified, taking into consideration a 1:3 and 1:6 noise levels: calibration point ratio, respectively.

Accuracy of the method showed satisfactory results, all values were less than 5% measured on the same day and less than 10% measured on another day. Correlation coefficients show high fitting of the linear on the calibration point and so the linearity of the curves is verified.

3.3. CAPILLARY ELECTROPHORESIS

3.3.1. METHOD OPTIMISATION

However, different capillary electrophoretic methods are known for determination of small molecule weight, polar thiols, mostly the from physiological and medicinal aspect more interesting thiols, like cysteine, homocysteine, glutathione, thiouracil, thiopurine compounds and / or their different derivatives were in the application front –line.

Wang et al. published a study about the determination of methimazole and other thiol substances by means of capillary electrophoresis with amperometric detection, [*Wang, et al.* 2000], but methimazole and especially its metabolites were not determined by CE before. Therefore and in hope of a better separation of the target compounds, we developed a nouvelle capillary zone electrophoresis method for the simultaneous analysis of methimazole and its metabolites.

Prior to practice, theoretical mobility of selected analytes was calculated with a help of self-made program. The program plotted the ratio of charge and molecule mass on the two third ($Q/M^{2/3}$) in the function of pH. From that diagram the pH range was estimated in which the analytes are fully or partly charged. For prediction pK_a values were needed, they were generated by Pallas.

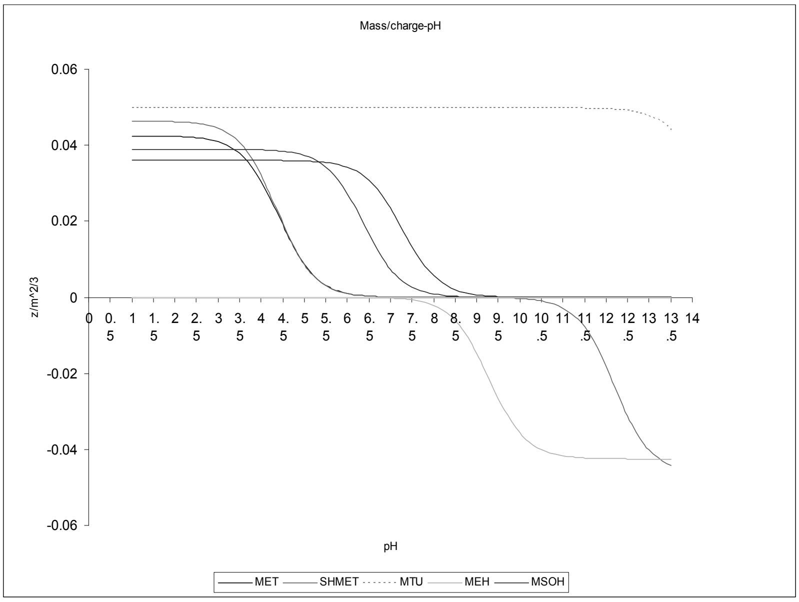


Figure 37. Theoretical mobility of methimazole and the selected metabolites.

CE measurements were carried out on a Beckman P/ACE 5510 (Beckman Coulter) capillary electrophoresis system equipped with DAD detector, the qualification and quantification was occurred at 230 nm and 255 nm. Fused silica, I.D. 50 µm, effective length 40 cm, total length 47 cm capillary was used as stationary phase.

The buffer system of the background electrolyte (BGE) was first selected. The buffer capacity should be the maximum in the estimated separation range to have similar mobility of the targets for run to run. On the other hand, the mobility of the buffer electrolyte should be in the range of the analyte's mobility to avoid the tailing of the peaks.

After selecting the buffer system, its optimal concentration had to be determined. The concentration of the buffer determines the EOF, current and peak symmetry. With increase of the concentration of running buffer the current increases since the resistance of the background electrolyte (BGE) decreases according to the Ohm equation.

As preliminary tests indicated, 10 mM di-sodium-hydrogenphosphate phosphate was found suitable for BGE electrolyte. 10 mM di-sodium-hydrogenphosphate showed proper current values (~65 µA) with good peak symmetry and in addition phosphate is suitable for application buffers even at extremely high pH values.

After the concentration of BGE separation buffer was determined, the pH of running medium was optimized.

According to the predictions, we started the experiments with low pH values: 10 mM phosphate at pH 3 was first investigated. 10 ppm standard samples were prepared from standard stock solutions by diluting with water and injected.

All peaks migrated with the EOF. Neither the repeated measurement with pH 1.6 phosphate showed better result in separation. Higher pH values in the range 5-13 were applied for further investigations.

Two peaks were obtained measured at pH 7: the two sulph-oxidised forms (MSOH and MSO2H) of methimazole got ionised and separated at the applied pH. It is important, because one of the expectations was, to be able to separate peaks that were co-eluted with HPLC. Other substances were migrated with the EOF, so neither ionisation no separation of the compounds was observed.

Measuring with pH 12 buffer had resulted 5 peaks in the electropherogram, but resolution of the peaks was not optimal, and MTU had migrated still with EOF. To achieve optimum pH further measurements were carried out. Changes in the electropherogram profiles at different pH values are displayed in figure 38. For a better overview, only the electropherogram of the four hard ionisable (MET, SHMET, MTU, MEH) substances are presented. Peaks are presented even on the converted migration scale.

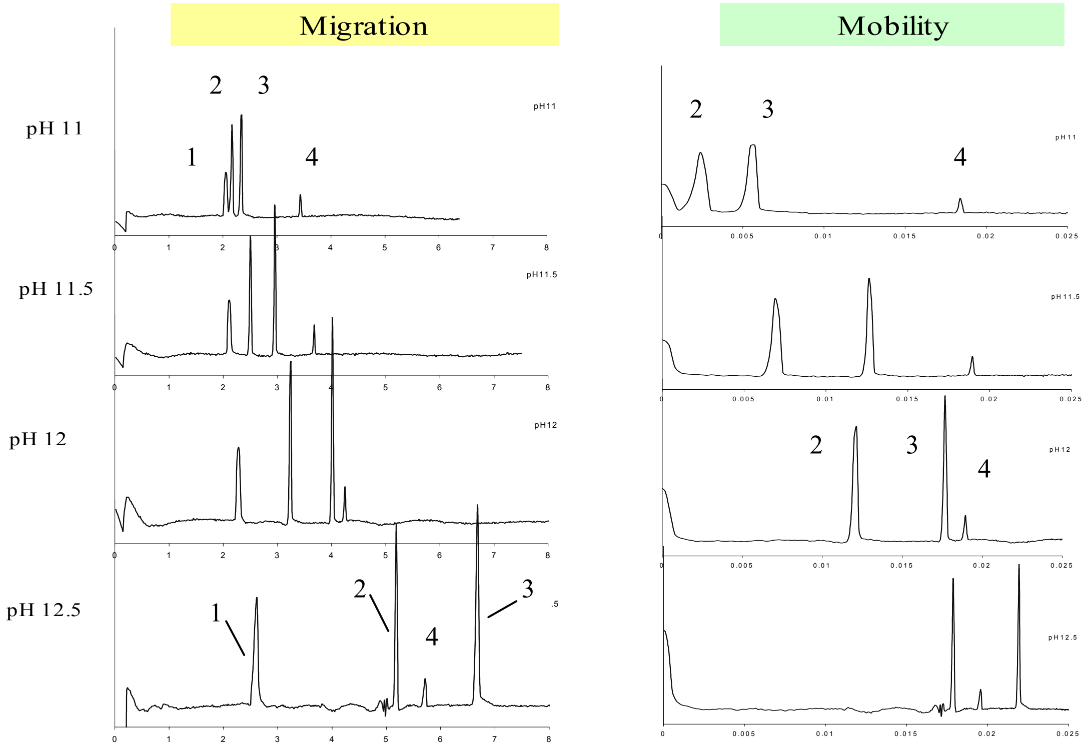


Figure 38. pH optimisation with the hardest ionisable substances (MET, SHMET, MTU, MEH) with comparison of the time-dependent (migration) and the time-independent (mobility) scales. Peak remarks: 1: EOF and MTU, 2: MET, 3: SHMET, 4: MEH

It is good to obtain, that at pH 11 also MEH got charged. Since eluting with the EOF is equal with the non-ionised status of the molecule, it can be pointed out, that separation of MET and SHMET from the EOF peak indicates their ionisation. But it is true, this ionisation is not complete, only partial. Buffer pH 13 caused too high current and so higher Joule heat developing which led to increased migration times and peak broadening causing lower signal heights, so it was not drafted in the graphs.

The strong pH dependence on the migration time (suddenly changes in the migration time) of the compounds (and the increased retention and resolution) indicate that the pK_a of the substances are found somewhere in this pH range. Effect of the pH on the mobility of the substances is showed bellow in the figure 39.

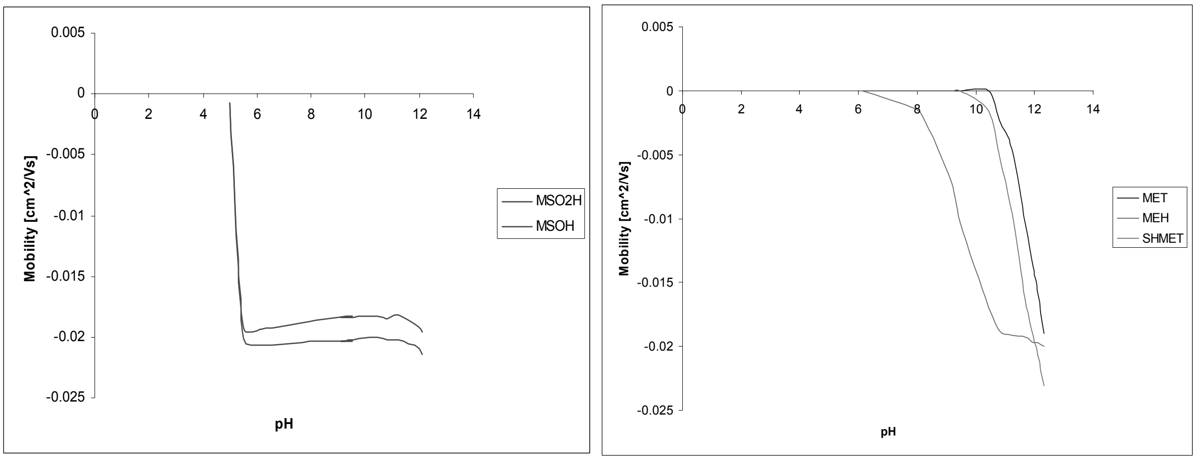


Figure 39. Changes in the mobility values of methimazole and its metabolites in function of the pH in the whole range. Curves are also suitable for the determination of the pKa values.

Graphics show clear, that MSOH and MSO2H are completely charged and could be separated above pH 6, what was not to achieve with the liquid chromatographic technique. Completely ionisation of MEH was observed above pH 10, while MET and SHMET were found as the weakest acids, pH>11.5 was needed for the ionisation of them. Finally buffer pH12.5 was chosen to be able to separate all ionisable target compounds simultaneously and with good signal to noise ratio. Separation of the substances was complete, co-elution or peak overlapping was not to observe.

Graphs are suitable also for determination (or at least for the visual estimation) of the pK_a values [*Lin et al.* 1993]. With the aid of the plots in figure above, one can easily determine the pKa of each analyte which is a weak acid by estimating the best value of μ_{A-}, the mobility of the fully deprotonated anionic form of each analyte at large pH ($>pK_a + 2$), of $\mu_{HA,}$ the limiting mobility of the neutral analytes, and then measuring the pH corresponding to $\mu_{ep}=0.5(\mu_{A-} - \mu_{HA})$ from the plot of μ_{ep} versus pH. Hence the pKa of each compounds is equal to the buffer pH at μ_{ep}=0.5(μ_{A-} - μ_{HA}). Table 22. lists the measured values of pKa for the substances of the interest.

Ionic strength was kept always at the same value for the measurements.

Table 22. Measured mobility values and the determination of the pK_as of methimazole and its metabolites.

Analytes	pKa	μ_{A-}	μ_{HA}
MET	11.8	-0.0166	0.0008
MSO2H	5.3	-0.018	0.0008
MEH	9.1	-0.0188	0.0009
MSOH	5.3	-0.0198	0.0009
SHMET	11.6	-0.0204	0.0009

This determination offers also a better opportunity to compare measured values with the predicted pKa values generated by Pallas, since chromatographic measurement was unsuitable for verifying the accuracy of the prediction process.

3.3.2. COMPARISON OF PREDICTED AND MEASURED pK_a VALUES

Capillary electrophoresis measurements presented an opportunity itself, to check directly the accuracy of the software used for the prediction of physical-chemical parameters. Data about the pKa and log D values were not found in the literature, except in the case of MET [*Stanovnik and Tisler*, 1964] and MEH [*Campi et al.*, 1965] therefore pKa values were determined by the developed CZE method and the results were compared to the predicted values. MEH value in the literature correlated well with both Pallas prediction and CE determination, but did not with the Sparc prediction. The Methimazole pK_a value in the literature correlated well with our measured value, but not with the predicted values by expert systems. The results were instructive, both prediction software had problems with our polar substances.

The most serious mistake of both programmes was the estimated basic character to the 3-N atom (-NH-) in the imidazole ring. It might have meant a basic characteristic of the compounds, what was not verified in none of the cases. Prediction for MET was totally failed; both programmes predicted basic characteristic of the 3N and an acidic character of the –SH group in case of the freeware. Pallas found no acidic group, what was then refuted.

MSOH and MSO2H were similarly totally false predicted. MTU was right calculated by both of the programmes for the acidic group but not for the 3-NH amide, since MTU was found not to be ionised between pH 2 and 13. SHMET was right predicted by Pallas for the acidic sulphydril group, while MTU was right predicted with both programmes for the =S group, both the basic character was false estimated.

3.3.3. ORGANIC MODIFIER

After determining the optimal concentration and pH of the BGE, organic modifiers were added into the background electrolyte at different concentration and their effect on the separation quality was investigated. Finally, the effects of applied voltage and temperature on the separation and current were investigated.

10 ppm standard solutions were prepared by diluting standard stock solutions and injected. 10 mM pH 12.5 phosphate was used as electrolyte. The effect of organic modifier acetonitrile on the separation is presented below in the figure 40. 0-20% organic concentrations were tested. Buffer pH was adjusted after adding organic solvent to the electrolyte. For a better overview only the outer and the middle peaks are plotted in both migration and mobility scale.

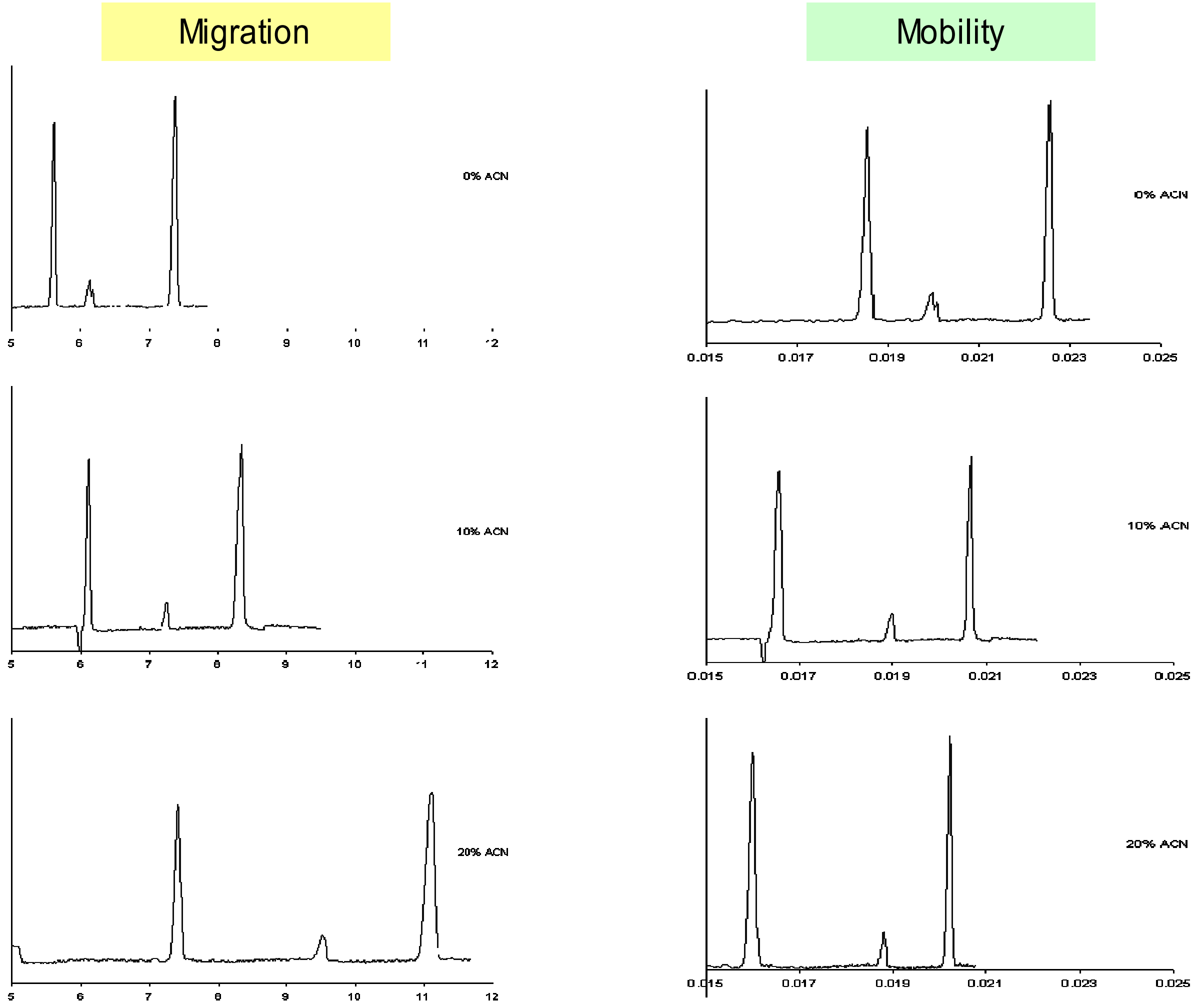


Figure 40. Effect of the organic modifier on the separation in comparison of the migration and the mobility scales. Acetonitrile was used as organic modifier in concentration range: 0 (above)-20% (below).

The use of organic modifier was not essential, since all peaks were separated well even without acetonitrile. But because of the potential application of the method for real samples, it was recommended to clear away the standard peaks from each other in order to avoid peak overlapping with the matrix components of the very complex fish homogenates, even if the time of the measure must be increased following peak broadening on the late eluted compounds. As registered electropherogram show, the use of acetonitrile in the buffer had no essential effect on the separation efficiency, but the standard peaks were better removed from each other causing better resolution values (and broader peaks). Other reason for application of organic modifier was a necessary decreasing of the high current value (~65 µA). Applying 20 % ACN in the electrolyte, this current value could reduce to ~55 µA.

However, the brutto run time was increased on the migration scale, decreased mobility was to obtain on the mobility scale plot.

3.3.4. OPTIMAL CONDITIONS

According to the above mentioned, the following parameters were found optimal for the determination of methimazole and its metabolites by capillary zone electrophoresis. The final separation protocol is summarized in table 23.

Table 23. Optimal conditions for the simultaneously determination of methimazole and its metabolites

Capillary:	Fused silica, I.D. 50 µm, effective length 40 cm, total length 47 cm.
Detection wave length:	255 nm for MET and SHMET and 230 nm for MEH, MSOH and MSO2H detector cell size: 100*800 µm.
Temperature:	Cartridge set to 30 °C.
BGE:	10 mmol/l di-sodiumhydrogenphosphate
Capillary preconditioning:	2 min pressure with 0.1 mol/L NaOH, then 3 min with BGE.
Injection:	Hydrodynamic injection of sample 50 mbar for 10 s (V_{inj}= 14 nl).
Voltage:	20 kV.
Replenishment:	Both separation vials and injection vial after each injection.
Capillary preconditioning: (new capillary)	Pressure flush with 0.1 mol/L NaOH for 30 min when it is used at first time
Storage capillary: (for next measuring day)	Pressure flush with 0.1 mol/L NaOH for 15 min before and after the measuring day
Storage capillary: (more then one week)	Pressure flush with 0.1 mol/L NaOH for 15 min followed by Milli-Q water for 15 min, then with air for 5 min.

Scale	Using the effective mobility scale transformed from electropherogram
Determination of concentration:	With five-point calibration curve in the working range between 2.5 mg/l and 50 mg/l determined every measuring day

Typical obtained electropherogram and the from that converted mobility scale plot with the optimised method parameters are presented in the figure 41.

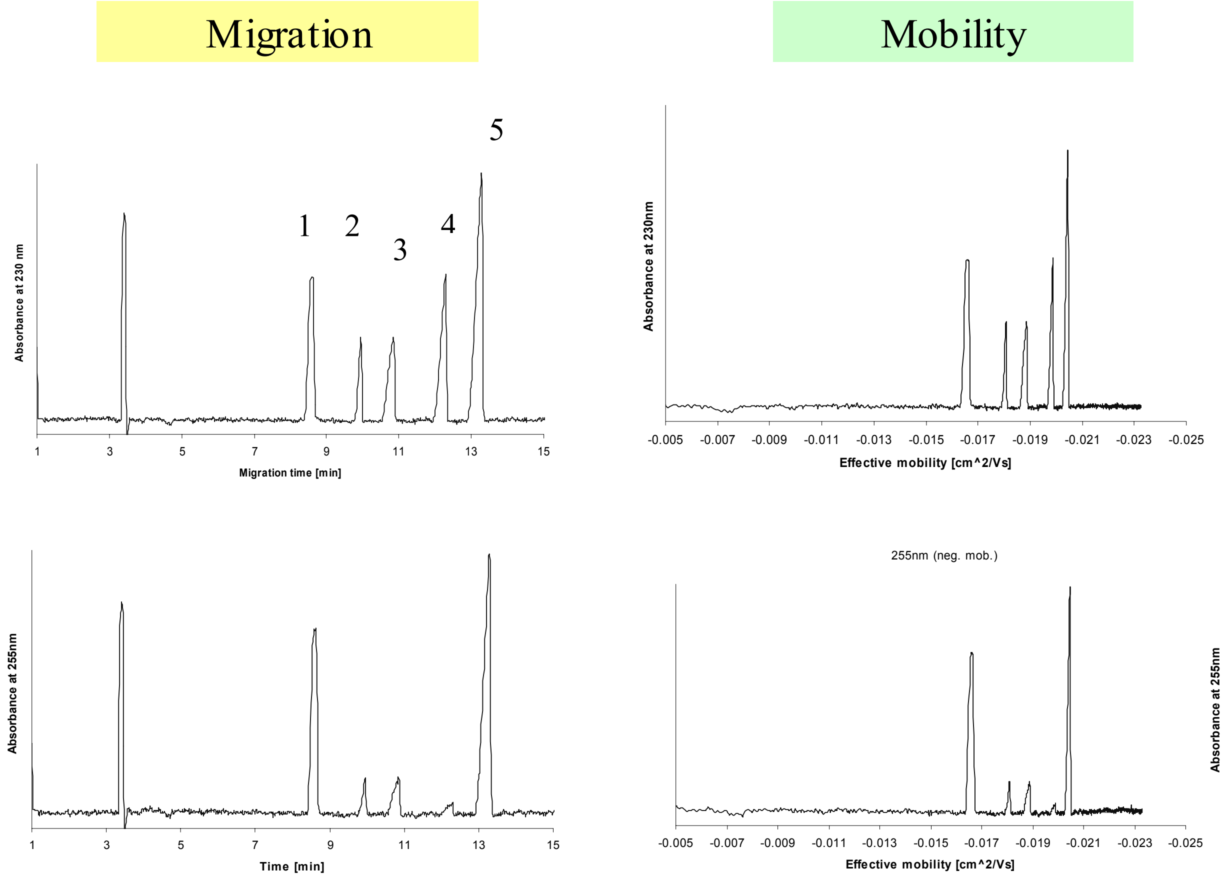


Figure 41. Typical obtained electropherograms with the optimised method on mobility and migration scales, detected at 230 nm (above) and 255 nm (below). Peak order was 1:MET, 2: MSO2H, 3: MEH, 4: MSOH, 5: SHMET for all plots.

Good separation of the target analytes were obtained on the plots. All resolution factors were higher than the required 1.5. Late peaks showed peak fronting on the migration plots, but this was reduced using conversion on to the mobility scale. Most important parameters of the quantification and qualification are detailed in the next point.

3.3.5. VALIDATION: PERFORMANCE CHARACTERISTICS OF CE USING UV DETECTION

The separation parameters were evaluated from electropherogram and normalised effective mobility scale.

QUALIFICATION

Resolution: R_s were calculated for the neighbour peaks step by step and the results are summarized in table 24.

Table 24. Resolution factors for the CE method.

Resolution factors		
Peak relation	Migration time	Effective mobility
MSO2H/MET	4.39	3.11
MEH/MET	2.73	1.78
MSOH/MEH	3.35	2.86
SHMET/MSOH	2.06	2.00

The maximal Rs value showed MSO2H/MET peaks in both case and the lowest was SHMET/MSOH with higher value than 1.5. The capillary was not overload in the investigated concentration range because the resolution was independent of the solute concentrations.

Precision of identification: The within-day repeatability of the identification parameters was calculated from five measurements at three analyte concentrations and summarized in table 25.

Table 25. Precision of the qualification with the CE method.

Compound	Migration time			Effective mobility		
	MT [min.]	RSD [%]	N	$\mu_{eff,norm}$ [cm^2/Vmin]	RSD [%]	N
MET	8.6	2.97	9131	-0.0166	1.45	27556
MSO2H	9.94	3.43	25294	-0.018	0.75	20736
MEH	10.84	3.14	11184	-0.0188	1.5	35344
MSOH	12.28	4.70	11915	-0.0198	0.38	69696
SHMET	13.27	3.28	10832	-0.0204	1.5	73984

The RSDs of MTs of the selected compounds were under 5 % showing an acceptable precision of this method. The RSD values evaluated with the effective mobility showed significant better results: RSD percentages were found between 0.75 and 1.5%. Even the measured theoretical plate number values showed clearly higher values (slimmer peaks) with evaluation using the converted effective mobility results, than it was to obtain in the case of the migration scale.

QUANTIFICATION

Linearity and working range: external calibration at eight points was carried out. Calibration points were: 2.5, 5, 10, 15, 25, 30, 50 and 100 µg/ml, respectively. Calibration standards were prepared by diluting stock solutions with water and injected in 4 parallel measurements.

The working range was established by the expected working range for the selected compounds. The measured calibration curves using electropherogram and effective mobility are shown in figure 42.

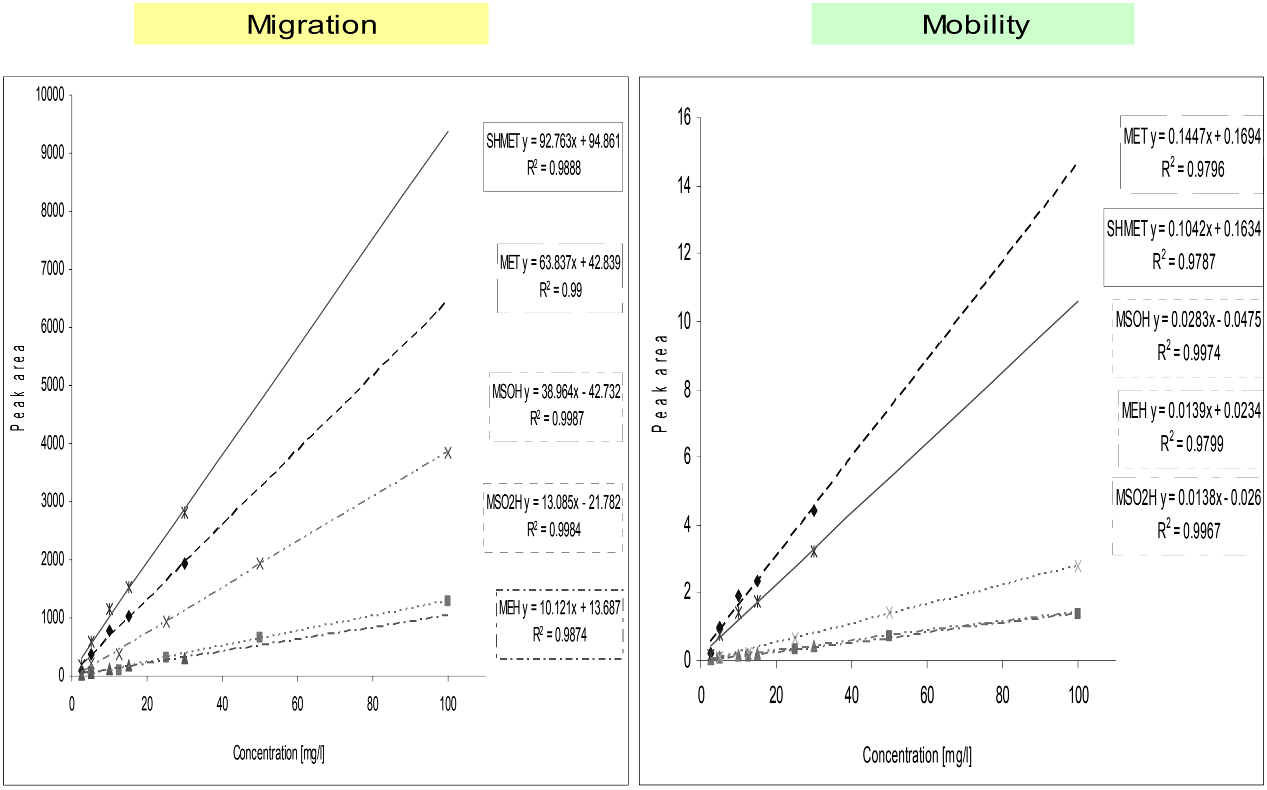


Figure 42. Calibration curves based on the peak areas of the peaks obtained both with migration and mobility scales

The most important validation parameters based on the migration (table 26) and the transformed mobility data (table 27) are summarized above

Table 26. External calibration parameters for quantification based on the migration data

Compound	Linearity			Sensitivity		Precision
	slope	intercept	r^2	LOD	LOQ	% RSD migration
MET	63837	42839	0.99	0.10	0.20	3.96
MSO2H	13085	-21789	0.9984	0.50	1.0	4.1
MEH	10121	13687	0.9874	0.64	1.28	3.35
MSOH	38964	-42732	0.9987	0.17	0.34	4.32
SHMET	92763	94861	0.9888	0.07	0.14	4.67

Table 27. External calibration parameters for quantification based on the mobility data

Compound	Linearity			Sensitivity		Precision
	slope	intercept	r^2	LOD	LOQ	% RSD mobility
MET	0.1447	0.1696	0.9796	0.10	0.20	1.71
MSO2H	0.0138	-0.026	0.9967	0.50	1.0	1.82
MEH	0.0139	0.0234	0.9799	0.64	1.28	1.94
MSOH	0.0283	-0.0475	0.9979	0.17	0.34	1.42
SHMET	0.1042	0.1634	0.9787	0.07	0.14	0.96

The linearity of standard calibration curves was confirmed by the values of the correlation coefficients. Correlation coefficients show higher fitting of the linear on the calibration points in the case of migration scale. Limits of detection and limits of quantisation were determined by taking into consideration a 1:3 and 1:6 noise levels: calibration point ratio, respectively.

Precision of quantification: The RSDs of the peak areas were calculated from five measurements and were fewer than 5% within a day for both scales, which is very good result for a quantification method in capillary electrophoresis. Significant changes in the precision values were obtained by using mobility scale for evaluation. RSD % decreased in all case, resulting excellent values: between 0.96 and 1.94, measured on the same day.

LOD and LOQ: The quantification limits for the target compounds were determined between 0.06 - 100 mg/L using electropherogram and normalised effective mobility scale.

It can be shortly summarised, that a novel separation method was successfully developed. No capillary electrophoretic method was known before for the simultaneous separation methimazole and its metabolites. Five of the six compounds of the interest could be separated with good performance, only the MTU got not ionised under the whole possible tested conditions. However, the free amino group seemed easily ionisable, finally it was neither to quantify nor to qualify. Three of the target analytes (MET, SHMET, MEH) could be ionised only at higher pH values, thus the both S-oxidised metabolites (MSOH, MSO2H) could be deprotonated even at neutral pH. The separation conditions were optimised and an optimal separation was achieved. Analytical performance characteristic was characterised in two different ways: on one hand side the results were evaluated on the basic of the conventional time dependent migration scale, and on the other side by application of a transformation into the time-independent effective mobility scale. Use of the effective mobility scale indicated some advantage, which are essential to be pointed out. It was presented that the use of the transformation to mobility shows extremely good reproducibility values in both cases peak areas and migration time (mobility). Much better theoretical plate numbers were obtained by using conversion to the mobility scale also.

3.4. SAMPLE PREPARATION

Isolation of drugs, drug metabolites and other relatively small organic substances from biological samples is complicated by the presence of a wide variety of macro and micro molecular substances in the samples. Macromolecules such lipids or especially proteins are the most uncertain in fish homogenised samples and complicate the analytical handling. Specific and sensitive sample preparation methods are needed for the selective extraction of the analytes from the complex matrix without co-extraction of substances causing disturbing in the chromatograms. Since abound lipids are easily removed from the aqueous fraction, proteins are still present in the inorganic fraction together with water soluble molecules e.g. drugs and metabolites. Protein removal could take place with various precipitating reagents and methods, but at the same way they have the disadvantage that some relevant substances maybe removed by occlusion. Traditionally liquid-liquid extraction has been used in most clinical or toxicological laboratories. However, recovery rates by liquid extraction are seldom complete. Liquid extraction tends to be slow and labor-intensive. More stringent environmental concerns are making the use and disposal of large amount of organic solvents more difficult. Solid-phase extraction is gaining in popularity for removal and isolation of drugs and other substances from biological samples. Compared to liquid-liquid extraction, solid-phase extraction offers the advantage of cleaver extracts, avoidance of emulsion formulation, speed, reproducibility and ease of operation and automation [*McDowall* 1989; *Majors* 1991]

Given the low concentration of drugs and so an in biofluids and the presence of many interfering substances in the sample matrix, an extraction–concentration step is always required. SPE is attractive for this step. Present-day analytical labs are concerned more and more with speed and efficiency as well as reliability.

We designed the sample preparation with knowledge of the above mentioned (and chapter 2.8.) deals of sample pre-treatment and of the observed chromatographic behaviour of the substances. It had also an importance to keep both solvent and time consuming at a minimum level and to develop an as much as possible simple method. Strict expectation from the sample preparation method is, the ability for the determination of all substance of interest simultaneously with higher than 70% recovery.

All measurement detailed in this chapter were carried out with the Pelkin Elmer HPLC equipment (s. details in the appendix) and with the optimised HPLC method fro the determination of methimazole and its metabolites.

A known method for the preparation of methimazole [*Moretti, 1993*] by liquid-liquid extraction was first investigated. We extended the list of the extracted substances and proceeded the method with our substances as follows: 2 ml standard aqueous samples containing 3, 30 and 75 ppm from each standard compound were prepared and extracted three times with 2 ml ethyl acetate. The supernatants were evaporated to dryness under nitrogen at 30°C and dissolved in 300 µl water prior to injection to the chromatographic system. Recovery values of the peak areas are shown in the figure 43.

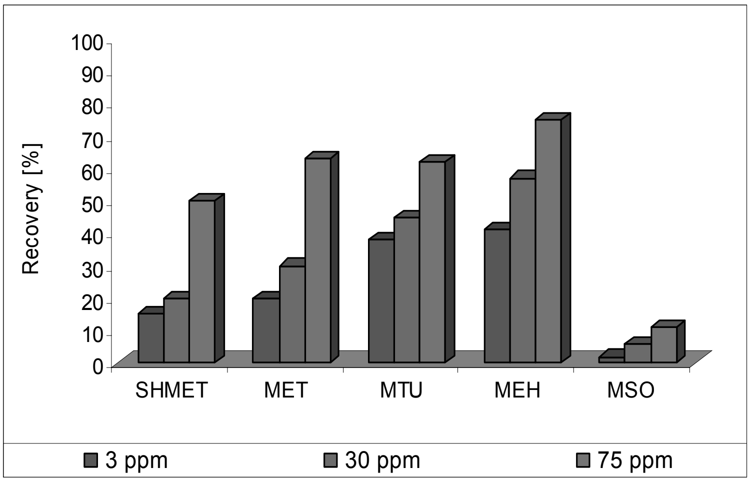


Figure 43. Recovery values of methimazole and its metabolites by liquid-liquid extraction. Standard solutions were extracted at three different concentration.

The published 59.7-76.9 % recovery values of methimazole in the concentration range between 0.2 and 8 mg/ml was not achieved. Methimazole recoveries were found significant lower than: between 20 and 63%. Other substances showed similar affinity to the organic phase then methimazole. However, MSO showed only a poor recovery (2-11%), based on the too polar character of the substance, the liquid-liquid extraction was found not suitable for the extraction.

The using of other organic solvents (e.g. acetone) for LLE was contraindicated because of insolubility problems of the compounds or one of them (SHMET and MSO). So, our developing activity got focused on to the solid phase extraction and related techniques.

3.4.1. RP-SPE

Oasis HLB

It was suspected from chromatographic experience, that there is no high capacity factor to achieve in the reverse phase modus, what actually evidence for an efficient enrichment of the substance on the particle surface of the cartridge. Some recently developing in column technology allowed the application of reversed phases in the SPE that are not available yet in the commercial analytical column form.

The basic structure of the OASIS cartridge series is the Oasis HLB column, which shows both hydrophilic and lipophilic character. Depending of the applied solvent it can be used both as polar or non polar reverse phase. Thanks to the N-vinylpirrolidon-divinylbenzole copolymer structure the Oasis HLB is a relatively hydrophilic extractive resin [*Waters; Y.F. Cheng et al.* 1997] with a wide pH range (0-14), which is mainly advantageous at preparation of (easily) ionisable compounds. We first studied the important extraction parameters with application of an Oasis HLB 60 mg, 3 ml extraction cartridge.

Breakthrough volume was determined by adding 1 ml standard 10 ppm mixture in each successively steps on to the cartridge. Aqueous standard stock solutions were mixed and diluted with water as the weakest elution strength solvent in reverse phase modus. The measurement was repeated in three parallel measurements. The Oasis HLB cartridges were conditioned with 2 ml MeOH and equilibrated with 2 ml water before application samples on to it. The drop down volumes were collected after each loading step and analysed with HPLC for the standard peak areas. The detected peak areas as the values of the theoretical 100% recovery of the loaded sample amount are presented in the figure 44.

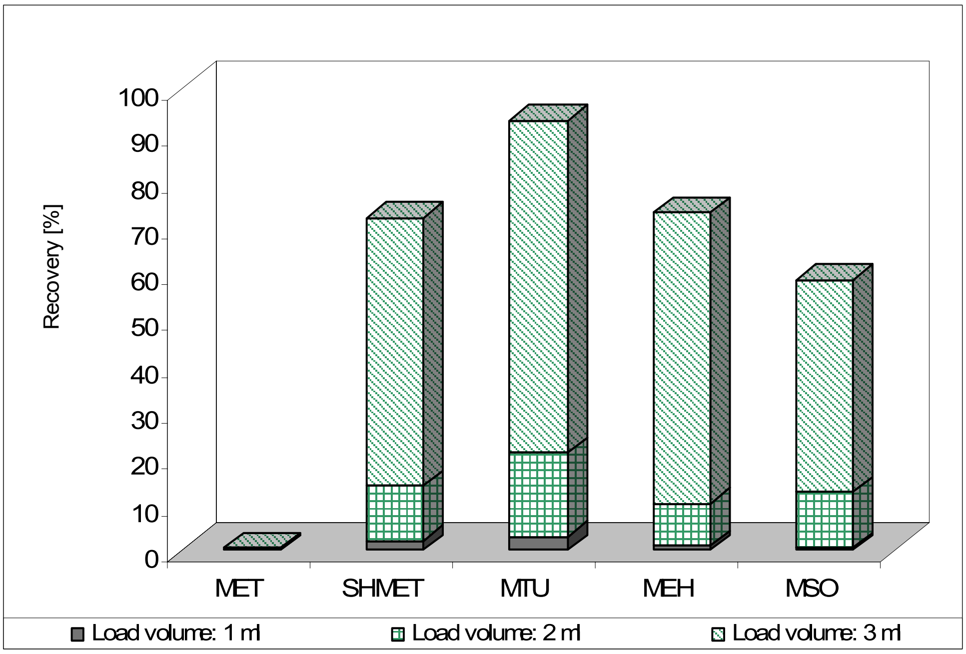


Figure 44. Determination of the breakthrough volume of the methimazole and its metabolites on the HLB extraction cartridge

As the figures represents and was expected, the highly polar substances showed poor retention on the RP surface in expectation of methimazole. However, V_B values were given less than 1 ml for SHMET, MTU and MEH, respectively, 1 ml for MSO and more than 3 ml for the mother substance MET, we defined 2 ml as the maximal loadable volume. Taking in to the consideration, that the maximal expected fish homogenate volume will be about 2-3 ml, and so the application of 2 ml samples on to the cartridge can theoretically prongs higher than 65-70% recovery rates for all substances, supposing that a nearly totally (~100 %) elution of the substances can be occurred, we examined onward with the determination of the ideal elution solvent strength and the determination of the concentration factors.

1 ml standard solution containing 10 ppm of each compound was given on to the top of the cartridge and was eluted with 2 ml 50 % methanol/water and 90% methanol/water solutions, respectively. The detectable standard peak areas were given as shown in the figure 45.

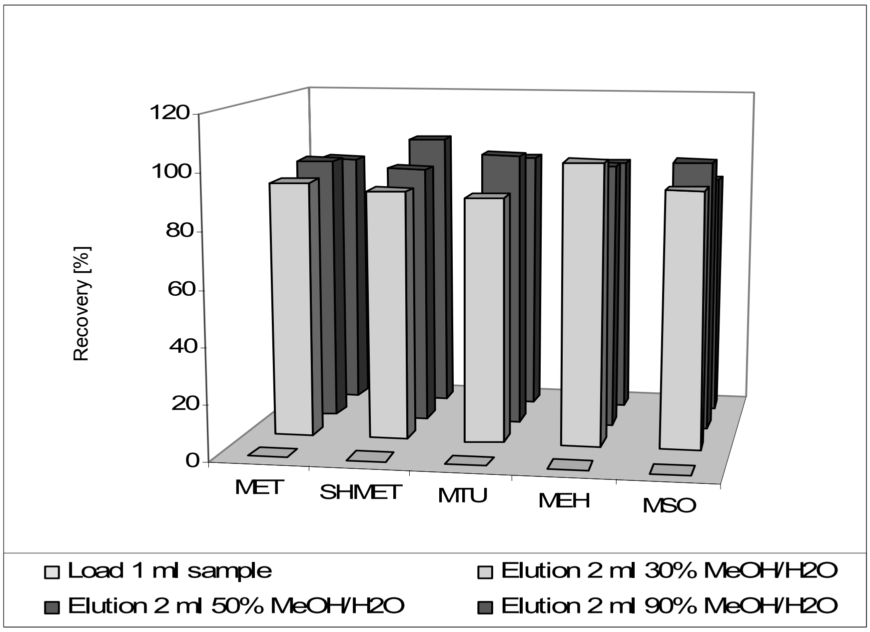


Figure 45. Determination of the optimal eluent composition and elution volume for the best recovery of methimazole and its metabolites on the HLB cartridge

It was found, that the presence of even a relatively small amount of methanol in the elution solvent is enough to elute all of the substances from the filling material of the extraction cartridge. 30% methanol content in the aqueous eluting solvent results as high recovery rates as does 90%. Using acetonitrile showed about 4-6% higher recoveries as did methanol, which is not to be wondered because of the higher elution strength of the solvent. The cartridge was eluted by 2 ml of the elution solvent, since application of 1 ml eluents showed 10-32% lower recoveries values in all case of compounds. Than samples were evaporated under a stream of nitrogen and resolved in 2 ml water before injection in to the chromatographic system.

It can be summarised at this point, that

- HLB cartridge shows only very low k′ values in the load step, so it is not suitable for the enrichment of the analytes
- Maximal applicable sample volume is 2 ml, but significant lowering of the metabolites recovery is predicted
- The substances were easily removable from the sorbent surface, even with weak eluent strength solvents (e.g. 30% MeOH/H_2O)

- The cartridge is useable only for clean up processes: either in modus with application of max. 2 ml of the sample onto the cartridge and than eluted in the first washing step with aqueous solvent containing methanol or in reverse modus, where the sample loading step acts as the elution step also.

Further solid phase extraction cartridges suitable for polar compound selection were tested to improve the enrichment potential and so the selectivity and the efficiency of the sample pre-treatment method.

3.4.2. GRAPHITIC CARBON PHASE (GCP)

GCP was an other tested RP-SPE phase, since according to the literature databases (s. chapter 2.8.2.) the very high hydrophobic character graphite sorbent can be successfully applied for sample preparation of very polar, extremely water soluble compounds. The polar adsorption arises from the surface oxygene complexes that are few in the number but interact strongly with polar compounds. GCP behaves both as non-specific sorbent via van der Waals interactions and ion exchange sorbent with potential for simultaneous extraction of neutral, basic and acidic compounds.

We tested an ENVI-Carb 3ml, 250 g, 120-140 mesh graphitic carbon cartridge (Supelco) with a surface area of 100 m2/g for the extraction properties.

The GCP cartridge was conditioned with 2 ml 80% methanol containing 20% CH_2Cl_2 and then equilibrated with 3 ml methanol and finally with 2 ml water before using.

Breakthrough volume (enrichment capacity) was determined by adding 1 ml standard 10 ppm aqueous mixture in continuous steps on to the column. Peak areas of the compounds of each drop down fraction were determined and the results are presented in the figure 46.

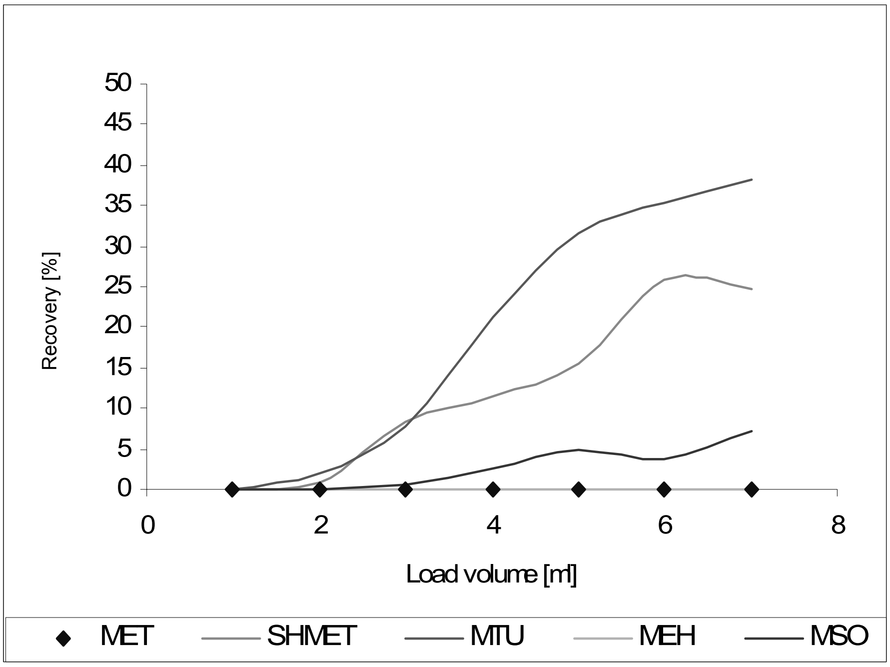


Figure 46. Determination of the breakthrough volume of the methimazole and its metabolites on the graphitic sorbent

As the graphic shows a significant increase in the applicable load volume was to be observed. After loading more than 7 ml samples there was neither MET nor MEH detectable in the pass through fractions. MSO was eluted only with the 4th ml and a mild increasing of the loadable sample volume of the MTU and SHMET was observed. 3 ml sample is applicable, with a less then 10 % recovery loss. On the basis of the solubility limitations of the compounds, solvents like methanol, acetonitrile, and 50% methanol-acetonitrile and 70% methanol-dichloromethane mixtures were tested for optimal elution solvent. 3 ml aqueous standard mixture was applied on to the column and eluted with 6 ml of above mentioned solvents. The found average recoveries are shown in the figure 47.

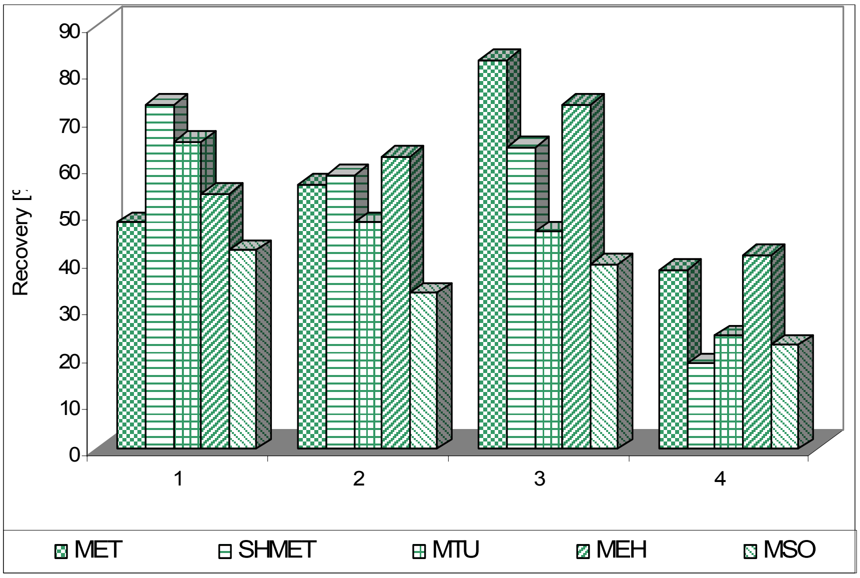


Figure 47. Recovery values of methimazole and its metabolites on the ENVI-Carb sorbent with using different solutions for elution. Solvent 1 was methanol, solvent 2 acetonitrile, solvent 3 was 50% methanol-acetonitrile and solvent 4 was 70% methanol-dichloromethane.

With standard samples it was required to achieve at least 80% recovery in case of all substances. The application of MeOH and ACN mixture provided more effective elution circumstances with the 46-82% recoveries than the solvents separately, but recoveries of MSO and MTU were poor (39 % for MSO and 46 % for MTU) and nearly in all case. The use of CH_2Cl_2 should be avoided, because the measured 18-41% recovery rates were far beneath the expectations. The results show, here was no optimal solvent found for the simultaneously elution of the compounds with satisfactory recovery values.

The conclusion from the above mentioned is:

- GCP cartridge can retain the investigated high polar compounds with high efficiency in the case of MET and SHMET and shows significant higher concentration potential for all substances, than does the HLB cartridge
- The maximal applicable sample volume is up to 7 ml
- In spite of the good concentration potential, the substances were eluted poorly
- The cartridge seems not to be suitable for application in our case because of the poor recoveries pf the MTU and MSO.

3.4.3. NP-SPE

Diol Phase

Normal phase is used to isolate polar compounds from a nonpolar sample matrix, so it was not easy to find an appropriate organic solvent to solve all of our compounds. Methanol and acetonitrile are suitable solvents, but also the elution strength of them is very high. Chloroform was published in literature as extraction solvent for MET and MTU (chapter 2.4.2.), but SHMET and MSO were found insoluble in it. In addition we avoided willingly the use of chloroform, since the goal of the method development was the building up of a method which is both bio medicinal-laboratory and health friendly in use. Only dichloromethane diluted with acetonitrile was found as the weakest solvent suitable for the normal phase application, however the solubility of MSO was very low in it. 100 ppm stock solutions of MET, SHMET, MTU and MEH were prepared in acetonitrile. Since MSO was self produced in basic aqueous solution, it had been had to change the solvent before. 2 ml 10 ppm MSO standard was prepared by diluting 1:100 the 1000 ppm stock solution. The 2 ml solution was evaporated to dryness under a stream of nitrogen and the substance was re-dissolved in acetonitrile. The standard mixture was then prepared from these stock solutions with diluting acetonitrile and dichloromethane to an end-concentration of 10 ppm for SHMET, MET, MTU and MEH and of 0.5 ppm for MSO. The end-concentration of dichloromethane was set either for 35% and 50 %. These solutions were given on to the cartridges. The extraction was carried out on Varian Bond Elut 2OH 3 ml, 150 mg cartridges. The SPE columns were conditioned with isopropanol and acetonitrile and equilibrated with 35% or 50% ACN/CH_2Cl_2 before application of the samples. The maximal applicable volume (V_B) was determined. The results are presented in the figure 48.

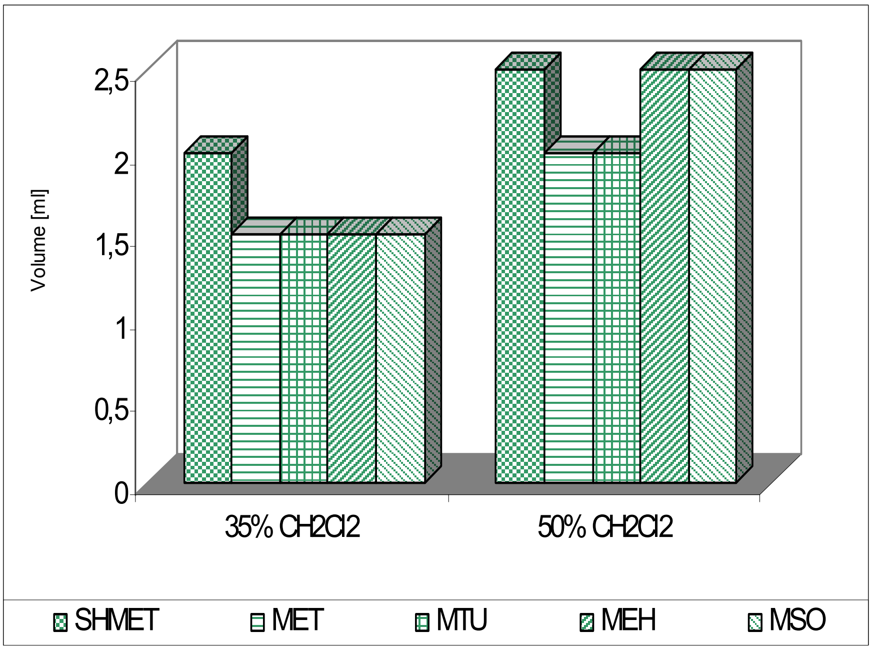


Figure 48. Determination of the maximal loadable sample volume for the diol phase

The maximal applicable sample volume was 1.5 ml with 35% and 2 ml with 50% presence of dichloromethane in the loading solution. 50% dichloromethane in the loading solution was found better for the adsorption and so for the enrichment of the substances. For recovery studies the adequate volume of standard samples were given on to the SPE columns with both 35 % and 50 % CH_2Cl_2 in the mobile phase (here equal with solvent for load sample). Cartridges were eluted with acetonitrile and methanol. Water was not applied because of the claim of easy and rapid evaporation prior to chromatographic analysis. Methanol was found suitable for the desorption of the analytes from the sorbent surface with good recovery values except in case of MSO with 61.4% recovery. The results with MeOH elution are shown in figure X. Up to 20% ACN can be added to MeOH to speed up the needed evaporation process after the extraction without observation any significant losses in the recovery rates. While 50% CH_2Cl_2 in the loading mixture gave nearly the same recoveries then 35 %, MET recovery was significantly better with the higher dichloromethane content in the sample loading solvent. The inconvenient solubility of MSO in acetonitrile and dichloromethane can be responsible for the poor recovery of the substance.

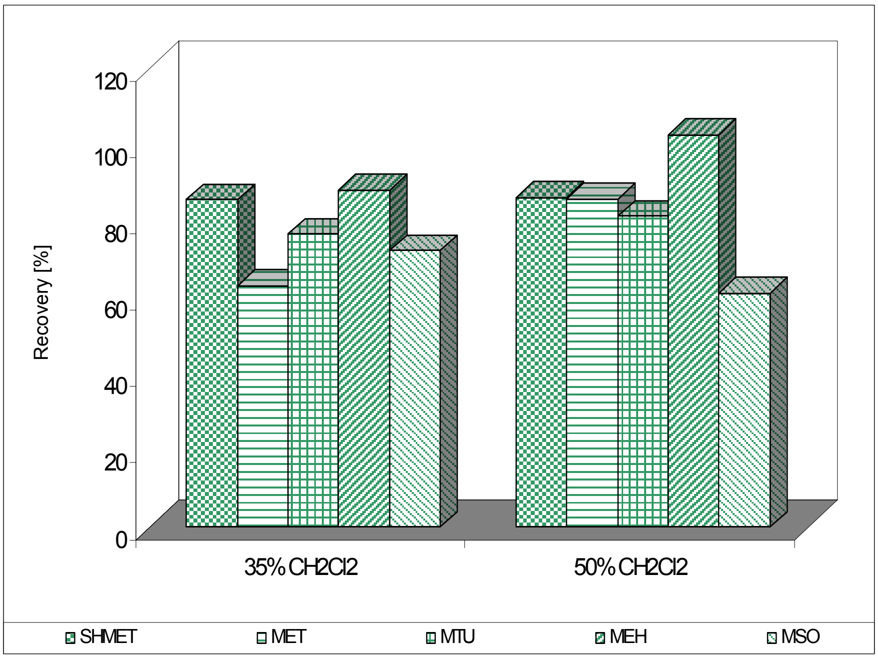


Figure 49. Recovery values of methimazole and its metabolites on the diol phase at two different CH_2Cl_2 concentration of the loading solution

We can draw the conclusions:

- normal phase 2OH SPE columns showed not the expected high retention of the analytes. The maximum applicable sample volume was 2 ml, which is hardly higher value than the reverse phase results
- MeOH is suitable to elute substances
- 50% ACN/CH_2Cl_2 is suitable for sample loading
- substances were easily to elute with methanol with good recovery rates, except MSO
- the cartridge was found not to be suitable for the determination, because of the poor solubility and elution-capacity of the MSO.

3.4.5. IONIC EXCHANGE SORBENTS

Fritz and Schmidt [*Schmidt & Fritz* 1993; *Schmidt* 1993] showed that neutral analytes as well as cations are retained by sulfonated resins provided the degree of sulfonation is not too great. On the basis of that and of the capillary electrophoretic experiences, anionic exchange extraction cartridges were tested for the retention capacity and elution characteristic. Oasis MAX 3 ml, 60 mg polymer based mixed-mode cartridges were used for the experiments. The use of silica based ionic exchangers is out of question, because very high pH values (pH>13) are needed to ionise the compounds. This can be only reached by using the polymer based (s. HLB column structure) mixed-mode cartridges. Extraction columns were conditioned with 2 ml MeOH and H_2O and finally equilibrated with 0.2 M NaOH (pH13). 10 ppm sample mixture was prepared from stock solutions, pH13 was adjusted with adding of 5M NaOH to it. Breakthrough volume (enrichment capacity) was tested by giving 5x1 ml of the 10 ppm standard solution on to the column and each drop down fraction was collected. Fractions were injected into the chromatographic system after neutralisation with 50% H_3PO_4/H_2O. The measured standard peak areas in the fractions are shown in the figure 50.

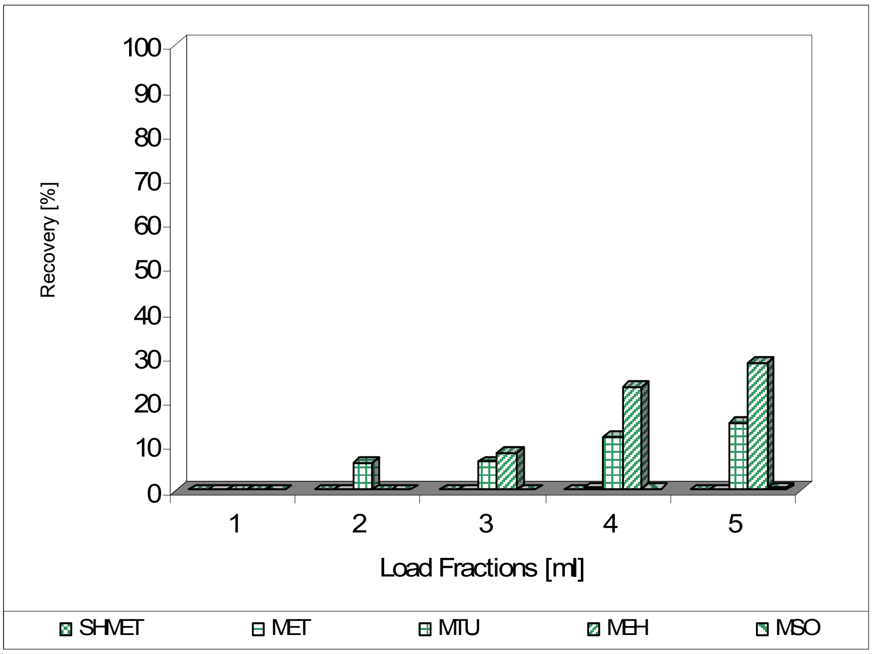


Figure 50. Determination of the breakthrough volume on the Oasis MAX cartridge. Recovery of the analytes in each loaded fraction was determined.

To determine optimal elution parameters, 1 ml 10 ppm standard solution (pH13) was given on to the cartridge and was eluted 4 ml 50% methanol/water, 70% methanol/water mixtures at different pH values. The pHs of the solvents were 1, 4 and 10 (adjusted with 0.05 M NaOH for pH 10 and 50% H_3PO_4/H_2O for pH1 and pH 4), respectively. The recovered substance peak areas in the eluents are shown in the figure 51.

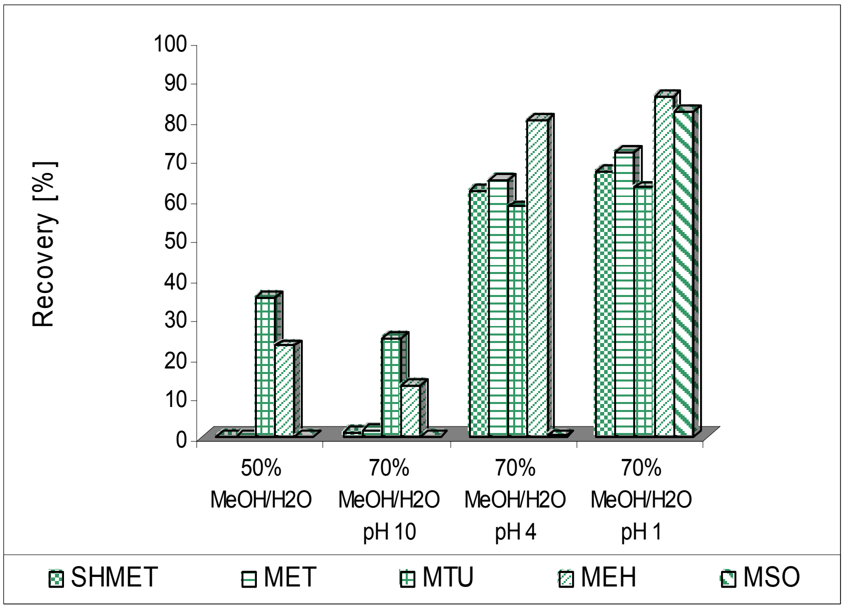


Figure 51. Determination of the optimal eluent solvent for the Oasis MAX cartridge. Recovery values of the target compounds were determined after elution with different solvents.

MAX-SPE examinations showed satisfactory retention of the analytes on the anionic exchange sorbent surfaces. The capacity factors were higher than in the case of the basic sorbent structure (HLB). More than 4 ml samples were applicable on to the column without any losses in the case of MET, SHMET and MSO, verifying a stronger interaction between the solid phase and the analytes. The MTU load ability was not better than with the HLB columns. 5% of the MTU was recovered in the second millilitre sample load step. In the third millilitre was also 8% MEH eluted and increased up to 12-23% in the fourth millilitre, which shows that no electrostatic but only the weak apolar-apolar interaction occurs between MTU/MEH and the ionic exchange-polymer surface.

Elution with 50% MeOH/H_2O showed very low recovery values of MET, SHMET and MSO. High washing out effect was obtained in the case of MTU (35%) and MEH (23%). This observation makes the use of the cartridge unsuitable for an efficient removal of the impurities with 50% MeOH/H_2O, which would be desirable to achieve more cleaner extract fraction for the chromatography. The elution strength of the 70% MeO/H2O pH10 solvent was found weaker than the elution strength of the 50% MeOH/H_2O, which can be explained by the higher pH value of the eluent and thus the lower pH changing effect on the cartridge material. Our expectation, that the compounds can be eluted in the pH range between ~7-10 was not verified. This could be meant also an increasing in the selectivity, which was also expected. MET, SHMET, MTU and MEH were eluted with 70% MeO/H2O pH4 mixture, but the recovery rates were not excellent 58-80% and MSO was not eluted at all. Simultaneous elution of all analytes were realised with using of 70% MeO/H2O pH1, however the recoveries were lower than required: 67% for SHMET, 72% for MET, 63% for MTU, 86% for MEH and 82% for MSO.

We can conclude:

- MAX solid phase is suitable for the enrichment of MET, SHMET and MSO, but not for MEH and MTU
- The maximal applicable sample volume is 3 ml
- Optimal solvent for the simultaneous elution of the substances is 70% MeO/H2O pH1
- the extraction does not provide high selectivity, because of the poor retention of MEH and MSO.

3.4.6. MSPD

On the basis of its simplicity and rapidity, MSPD methods were tested in expectations to saving extraction time, increase the clean up efficiency and the recoveries due to the better blending of the sample and the solid phase particles. 50% blend of normal and reversed phase bulk materials were prepared to achieve more effective retention of the complex matrix substances, resulting improved extract purity.

8 ml glass tubes were fritted with 400 mg glass wool and filled with 0.3-0.5 g Florisil or with Florisil containing 50% Nucleosil C18 column packing material thoroughly blended with 2-3 mg of standard substances (spiking). 300 µl 1000 ppm MSO (0.3 mg MSO) solution was pipetted to the blended solids and eluted with different solvents and mixtures. Solvents were chosen considering the solvent compatibility even with the SPE applications. Because of the high time-consumption of the evaporation process of the eluted fractions after the elution, we maximised the applicable eluent volume: the spiked blends were flushed with 10 ml solvent, vortexed for 5 min and than eluated. Eluted fractions were evaporated under a stream of nitrogen and all samples was dissolved in water prior to chromatographic analysis. The used MSPD systems, the eluents and the measured recovery rates are listed in table 28.

Table 28. Construction of the MSPD systems and the recovery rates of the analytes

Sorbent	Eluent	Recoveries [%]				
		SHMET	MET	MTU	MSO	MEH
300 mg FLORISIL	CH_2Cl_2	20	70	25	2	36
300 mg FLORISIL	50% ACN/ CH_2Cl_2	98	88	48	16	70
300 mg FLORISIL	ACN	105	90	98	34	70
300 mg FLORISIL	MeOH	130	98	80	45	77
300 mg FLORISIL	50% MeOH/H_2O	105	88	90	70	68
300 mg FLORISIL	H_2O	38	36	35	58	29
400 mg 50%FL-C18	MeOH	82	74	67	55	62
400 mg 50%FL-C18	50% ACN/ CH_2Cl_2	105	99	87	24	78

As it was expected, using eluents containing acetonitrile, methanol and water one by one or in mixture showed good recovery values. The hoped good recoveries with using dichloromethane were not observed, which could have been advantageous for a more effective clean up of the very complex matrix, especially in the case of the 50% FL-C18 MSPD column in coupling with SPE methods. Good elution efficiency in this case would have been raised the opportunity of both polar and apolar clean up steps (in combination with SPE) resulting cleaner chromatograms with less peaks in the chromatogram and less interference problems. As the results show, only 50 % MeOH/H_2O was suited to elute all of the substances simultaneously from the polar florisil sorbent. In spite of using standard substances without matrix components, the recovery rates were not fascinating in the case of MSO and MEH recovery (68 and 70%). Neither the use of 50 % MeOH/H_2O was optimal for a low time-consuming method: the evaporation of 10 ml of that extraction requires too much time, however the method is very simple to carry out. The capability of the method with matrix samples is presented below in chapter 3.4.8.

3.4.7 ON-LINE SPE

On-line coupling of a C4-reverse phase Alkyl-Diol Silica (LiChrospher ADS-RP4 4x4 mm, Merck GmbH, Germany) cartridge with the Atlantis column was constructed (Fig. 52.). The goal of the experiment was the study of the online clean up potential of the system (especially for removing proteins from the sample). Even the enrichment potential of the short chain (C4) adsorbent was examined. First the retention time of both proteins and methimazole and its metabolites on the SPE-cartridge was determined. To determine this time, the analytical column must had been disconnected from the system, the injected samples were passing the ADS cartridge and then directly the DAD detector. The ADS column was washed and equilibrated with methanol and 5% methanol/water mixture prior to injection. To simulate the retention behaviour of the proteins of a fish whole body homogenate, 3% albumin solution was injected. Retention time of the albumin solution was compared to the retention time of methimazole and its metabolites.

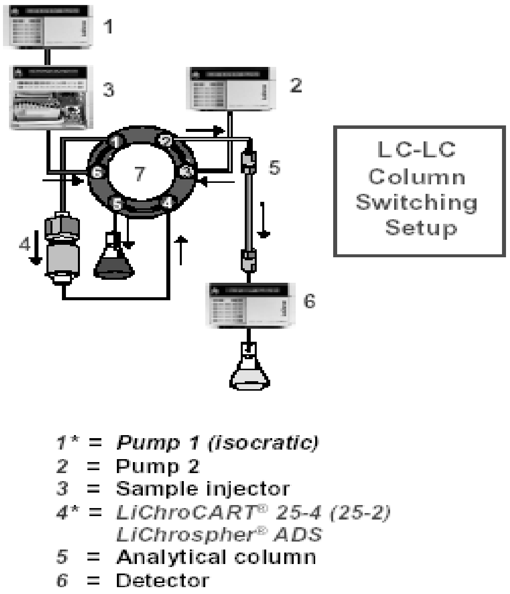


Figure 52. On-line SPE system setup

2% methanol/water containing 0.01% H_3PO_4 was used to elute the substances. The pH of the eluent was pH3 to keep the proteins in their isoelectric range. The linear flow rate was 0.8 ml/min. Albumin was detected at 280 nm, while methimazole and its metabolites were detected at 220 nm. Sufficient difference in retention times of the albumin and the analytes was not to observe, the methimazole and its metabolites were eluted in one peak. Resolution factors were less then 1.5, which was far away from the required $R_s > 20$ value (equals with the condition $t_{r,prot} << t_{r,analytefor}$) for the ADS application. The measurement was repeated with the 100% water (weakest eluent) containing 0.01% H_3PO_4 as eluent, while other parameters were the same. No considerable change in the retention behaviours and the measured resolution factors were obtained. As only device, the linear flow rate was decreased in the following examination steps. 0.5 and 0.1 ml/min flow rates were applied to increase the resolution of the albumin and the methimazole (and metabolites). 2% methanol/water containing 0.01% H_3PO_4 was used as eluent. The presence of 2% methanol was essential to change the conformation of the proteins to set conjugated substances free. It was mainly interesting from the view of the thyroid hormone analysis, the other main research topic of this thesis. The obtained elution curves with different flow rates are presented in the figure 53.

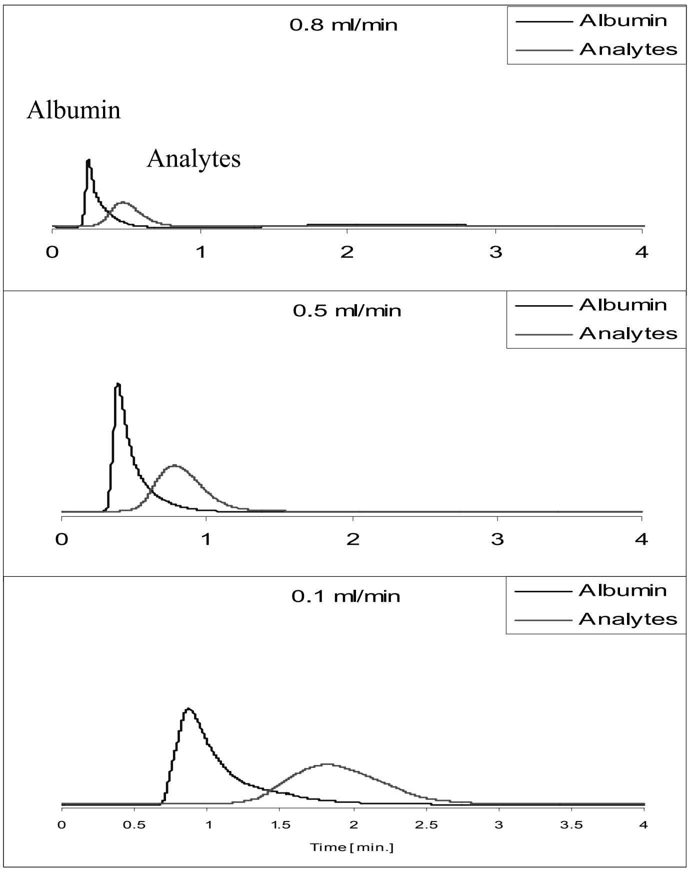


Figure 53. Obtained elution profiles of the target substances compared to the elution profile of albumin using on-line SPE extraction technique at different flow rates

The decreased flow rates caused no improvement in the retention difference. The condition, that $t_{prot} << t_{analyte}$ was not fulfilled in neither of the cases. R_S values were 0.44 with 0.8 ml/min, 0.46 with 0.5 ml/min and 0.70 with 0.1 ml/min flow rate, respectively. However, there are LC pumps available with lower solvent delivery capacity, the observed poor retention values of the analytes and the poor resolution values do not hold out promises of achieving the required resolution values.

It can be concluded, that:

- the on-line system is not suitable for the on-line clean up process of the samples
- the short chain (C4) solid phase does not provide satisfactory retention of the tested analytes.

3.4.7. PROTEIN REMOVAL FROM THE SAMPLES

High amount of proteins are present in a fish matrix and they means the most problematic impurities of the sample for the chromatographic system. Protein chains can easily coiled on to the surface of the column solid phase particles causing blocking in the column. In simple cases it can cause higher back-pressure or varying in the retention time, but after few number of sample injection totally column blockage can often be occurred. Various methods are available for removing proteins from the sample solution. [*Polson et al.* 2003]

Precipitation methods using organic solvents (min. 50%) or high salt concentration are simple to carry out and are efficient. Dialysis, gel permeation chromatographic techniques are further opportunities.

In our method development we investigated ultracentrifugation and solid phase extraction for protein removal in addition to the precipitation with organic solvent. Protein concentrations were determined by the means of Lowry´s method. A BioRad RC DC Protein Assay kit was used for the rapid determination of the total protein concentration.

The microfuge tube assay protocol was carried out as follows:

25 µl of samples was pipetted into microfuge tubes. 125 µl RC Reagent I was added into each tubes and the mixture was vortexed for 1 minute at room temperature. 125 µl RC Reagent II was added into each tube and the tubes were centrifuged at 15000 x g for 5 minutes. The supernatant was discarded, the liquid was completely removed from the tubes. 127 µl Reagent A´ (5 µl of DC Reagent S added to each 250 µl of DC Reagent A) was added to the tubes, the

mixture was incubated at room temperature for 5 minutes and than vortexed well. Finally 1 ml of DC Reagent B was added to the solution and was vortexed immediately. After 15 minutes incubation, the absorbances can be read at 750 nm. Parallel to the samples 0.2 – 1.5 mg/ml standard albumin solution were prepared and measured, since standard curves were prepared each time the assay is performed.

To determine the average total protein content of the fish samples, six fishes were homogenised in water and were determined individual.

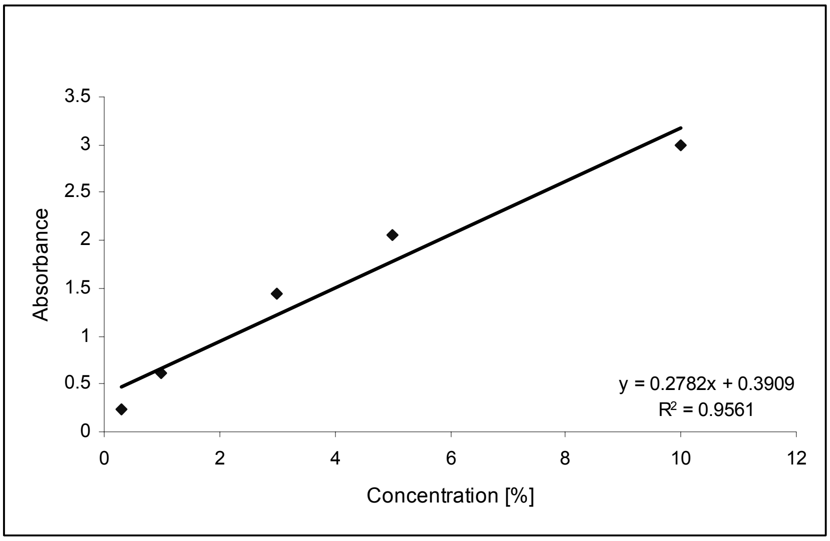


Figure 54. Albumin calibration curve for Lowry´s method to determine protein concentration

Protein content of the fish whole body homogenate was found <u>10-30 g/l.</u> This high value also verified the need of an efficient sample pre-treatment.

High speed centrifugation and SPE methods were also tested for protein removal efficiency. The high speed centrifugation (s. details on the next page) was carried out with Beckman L-60 ultracentrifuge. Oasis HLB cartridge was used for SPE tests.

0.5 and 3 % albumin solutions were prepared (n=2) in water (100% albumin) and in 10-50% MeOH/H_2O for testing effect of organic solvent content on the protein precipitation. Each solution was left to stand for 30 minutes at room temperature, than was centrifuged at 15000 x g for 10 minutes and albumin concentrations were determined. The results are shown in table 29 and are given as values referring to the total (100%) albumin value defined above.

Table 29. Protein content of the mixture containing 0-50% methanol

% MeOH	0.5 % albumin	3 % albumin
0	100%	100%
10	89%	92.1%
20	92%	96%
30	72%	64%
40	48%	46%
50	51%	44%

For testing centrifugation and solid phase extraction on the protein removal efficiency three parallel fish samples were prepared in water and another three fishes in 10% $ZnSO_4$ solution (promotes precipitation) and measured for average protein concentration (100%). The samples were centrifuged by 80.000 x g for 20, 45, 60 and 90 minutes to achieve the most efficient removal of the proteins and also the cell debris from the supernatant. Maximal rotation speed (80.000 x g) was set taking the speed resistance of the centrifuge tubes into consideration. Protein contents of the samples were determined and the consistence of the supernatant was observed. All fractions were collected and protein content was determined for each, taking values refer to the above mentioned total protein values.

Then 60 minutes centrifuged samples were extracted with the HLB cartridge. 2 ml of centrifuged samples was given on to the column. Cartridges were washed with 2 ml water and than eluted with 50% MeOH/H_2O. All eluted fractions were measured for protein concentration. The results are presented as recovery values referring to the 60 minutes centrifuged sample values as 100%. The results are presented in the table 30.

Table 30. Protein content of the standard sample after centrifugation for different time and after SPE extraction, both methods was tested with water and with high salt concentration

	water	**10 % $ZnSO_4$**
Centrifugation		
20 min.	38 %	26 %
45 min.	28 %	18 %
60 min.	25 %	15 %
90 min.	20 %	18 %
SPE		
sample load	40 %	23 %
washing	23 %	16 %
elution	20 %	30 %

The results show, that a minimum 45 minutes high speed centrifugation alone or in combination with SPE is very efficient in removal of the proteins from a complex biological matrix. However, adding 10% $ZnSO_4$ to the sample caused significant higher removal efficiency. The organic content of the sample should be plant to be higher than 40% at the extraction of the homogenates to achieve a lower protein co-extraction from the matrix.

Summarised we can point it out, that more than 90% of the proteins are removable with the combination of the above mentioned procedures.

3.4.8. INVESTIGATION OF MATRIX EFFECT

Matrix effect on the extraction efficiency of the tested SPE cartridges was studied by the extraction of untreated and spiked (10 ppm) fish whole body homogenates. The fishes were stored at -70°C until proceeding. Fish samples (n=3) were homogenised by a Schütt Homgen homogenator at 4°C. Solvents for homogenising (loading) and for elution of the samples were selected taking the optimal both sample load and elution conditions for each cartridge individually (s. experimental report above with standard solutions) into considerations. Each homogenising solvent was spiked with each standard substance in 10 ppm concentration before homogenising fish into it. Homogenised samples were centrifuged by 32.000 rpm for 60 minutes and than extracted with the tested SPE cartridges. Oasis HLB, MAX, DIOL and Graphite phases were tested. Optimal conditions were reported for each column above in the SPE phase overview.

Fish samples for MSPD experiments were freeze-homogenized: the freeze (-70°C) fish bodies were mortared in liquid nitrogen and mixed with the 50% FL-Nucleosil C18 phases prior to filling them into the glass tubes. Elution was carried out with 50% MeOH/H_2O. The total recovery of the analyses was determined for each procedure and the results are demonstrated in the figure 55.

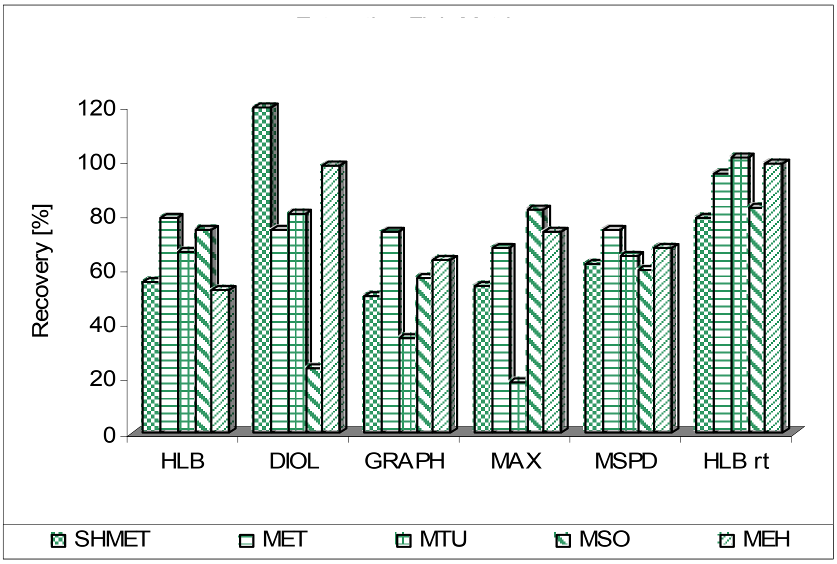


Figure 55. Comparison of the extraction efficiency of the tested SPE methods with spiked fish samples. HLB rt: load and elute technique with the HLB cartridge.

Experiments showed that the matrix had effect on the recovery of the standard substances. About 10-20% decreasing of the yields from matrix was obtained. None of the "classical" SPE applications showed good efficiency on the sample preparation: there was found no suited cartridge and method for the simultaneous determination of the target analytes. Because of the polar and different character of the substances, highly specific method for sample preparation was not found. Only "load and elute" methods without retention of the target analytes were found suitable, however even these highly polar substances can be enriched and selectively eluted with SPE methods. In our case the requirements of the simultaneous elution was not fulfilled. Good simultaneously yield of the substances were obtained with the MSPD method eluted with 50% MeOH/H_2O, but the calculated recovery values were between 62 and 82 %, which are not acceptable according to the EPA requirements. Another problematic side of the method was the relatively impurity of the sample extracts and the time-consuming of the homogenising step. HLB extraction with load and elute in one step application was found as only sample preparation method suitable for the simultaneous determination of all target compounds with higher than 70% recovery values. This method was validated and used for the routine application.

3.4.9. THE OPTIMISED SAMPLE PREPARATION METHOD

The sample preparation for the analysis was found optimal as:
Adult fish and larvae samples were stored at -70°C until processing. Weight of each fish was determined and adult fishes were homogenized in 2 ml 60% MeOH/H_2O solution by a Schütt Homgen homogenator with 15 ml conical glass vessel at 4°C. Homogenates were poured into 8 ml centrifuge tube. The debris was washed with 0.5 ml 60% MeOH/H_2O and it was added to the centrifuge tubes also. The extract was centrifuged at 80.000 x g for 45 minutes. The supernatant was diluted with 2.5 ml water to lower the elution strength of the solvent and this was applied onto the top of the HLB cartridge and pressed through. HLB cartridges were conditioned with 2 ml MeOH and equilibrated with 2 ml 60% MeOH/H2O prior to application of the samples. The drop-down fraction was collected, and 2 ml ACN was added to that to accomplish the protein removal. Samples were left to stay for 30 minutes at room temperature and centrifuged at 20.000 x g for 15 minutes. Supernatant was filtered through a Millipore Millex HS 0.2 µm filter, was evaporated under a stream of nitrogen and dissolved in 300 µl water. 10 µl sample was injected into the chromatographic system.
Not satisfactory resolute and overlapped peaks derived from the fish matrix were obtained in the 7-10 minutes chromatogram range using the optimised HPLC method at pH>5. It was given problematical, because two of the substances of the interest (MH and MET) were eluted within this time range. Due to the pH ruggedness of the retention times of methimazole and its metabolites, the pH of the eluent value was adjusted to pH 3.8 to change retention behaviour of the co-eluted peaks and without any changes in the retention of methimazole and metabolites. The resulted changes in the chromatogram outfit are introduced due to the example of a methimazole spiked fish sample in the figure 56.

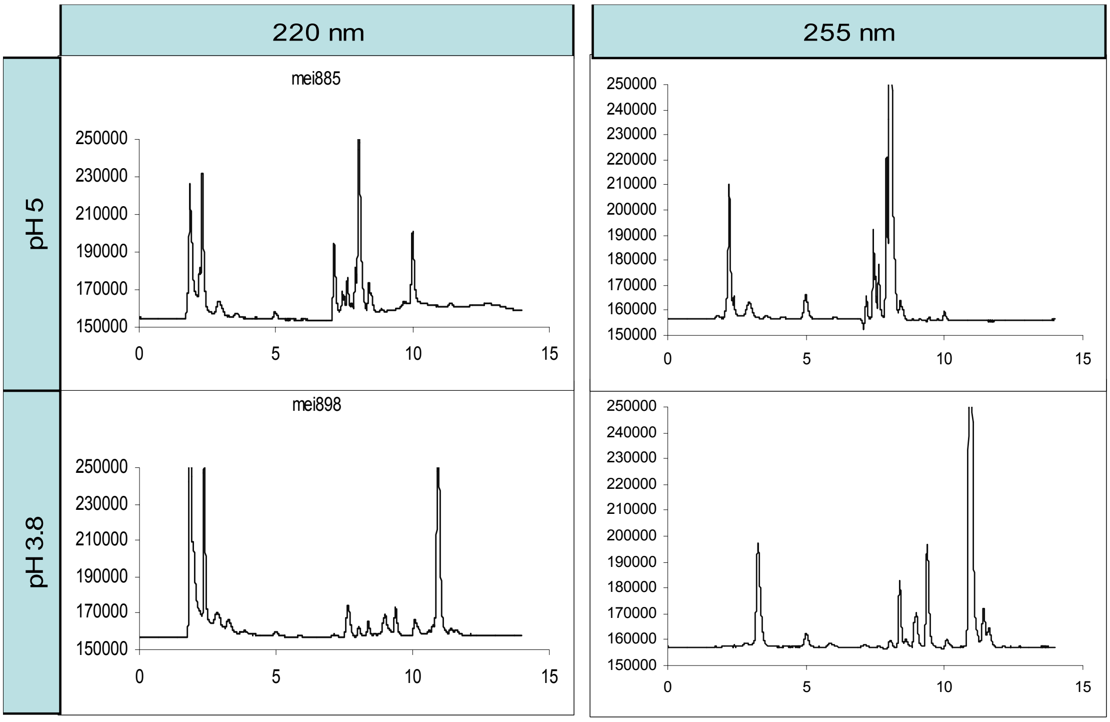


Figure 56. Effect of the eluent pH on the prepared fish matrix extract. Better resolution of the matrix peaks was obtained at lower pH.

ACCURACY OF THE WHOLE ANALYTICAL METHOD

Accuracy of the whole analytical method was tested by adding standard substances to the solvent, that was used for homogenise the fish samples. Five parallel samples were spiked at three different concentrations. The spiked values were 0.5, 3 and 10 µg/ml. Standard recovery values were determined by HPLC after carry out of the sample preparation for each sample. The measured recovery values and the relative standard deviations are shown in the table 31.

Table 31. Recovery values for the whole analytical method

Recovery of the whole analytical method [%] ± RSD [%]					
	MET	SHMET	MTU	MSO	MEH
0.5 µg/ml	79.9 ± 6.9	87.4 ± 6.6	88.7 ± 5.9	76.6±8.3	93.4 ± 5.4
3 µg/ml	86.2 ± 4.8	89.8 ± 6.7	94.2 ± 7.3	81.3±5.4	101.3 ± 6.2
10 µg/ml	97.4 ± 5.6	78.2 ± 12.7	102.1 ± 8.6	83.6±6.9	98.2 ± 7.6

Recovery values showed good yields of the added analytes from the complex fish matrix. Recoveries fulfilled the EPA requirements (w>70%) for all compounds; this and the elimination of the matrix peak overlapping problem make the usage of the method possible for routine analysis of fish samples.

TYPICAL CHROMATOGRAMMS OF EXTRACTED ADULT AND LARVAE SAMPLES

Typical chromatograms of extracted spiked and extracted blank fish samples detected at 255 nm and 220 nm are shown in the figure 57. pH 3.8 phosphate was used for the elution, other chromatographic parameters were described in chapter 3.2.9. On the basic of the outfit of the observed chromatograms it can be concluded, that the clean up process of the very complex biological samples was effective for observing clear and both qualitatively and quantitatively easy evaluable chromatograms at both detection wavelength. As it was expected, observed chromatograms detected at 220 nm contained more peaks, but peak overlapping of the analytes of the interest was not observed, all resolution values were higher than 1.5.

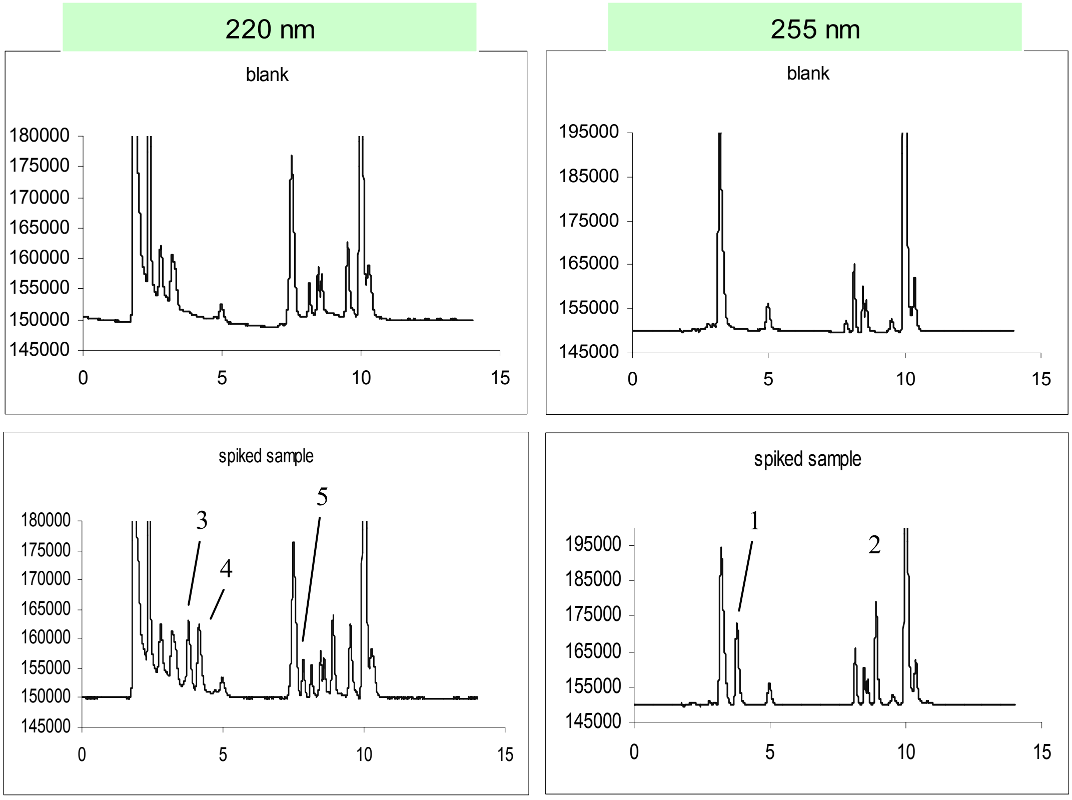


Figure 57. Typical chromatograms obtained after extraction of blank (above) and spiked (below) fish samples. Peak remarks are: 1: SHMET, 2: MET, 3: MTU, 4: MSO, 5: MEH

Fish embryonal extracts showed different chromatographic outfit than had adult samples. It was suspected, that early developing state animals are less complex, than the adults. The registered chromatograms showed the opposite results. However, the larvae were extracted with the same method as the adults, the resulted chromatogram outfits were different from them. Adult chromatograms seemed cleaner with normal good separated peaks. Opposite to that, larva-extracts contained more water and/or water/methanol soluble compounds consequently more (noisy) peaks in the 5-10 minutes elution range. Some overlapped and nearly co-eluted peaks made difficult the identification of MEH and the main compound MET. Typical chromatograms of blank and spiked larva samples are presented below in figure 58.

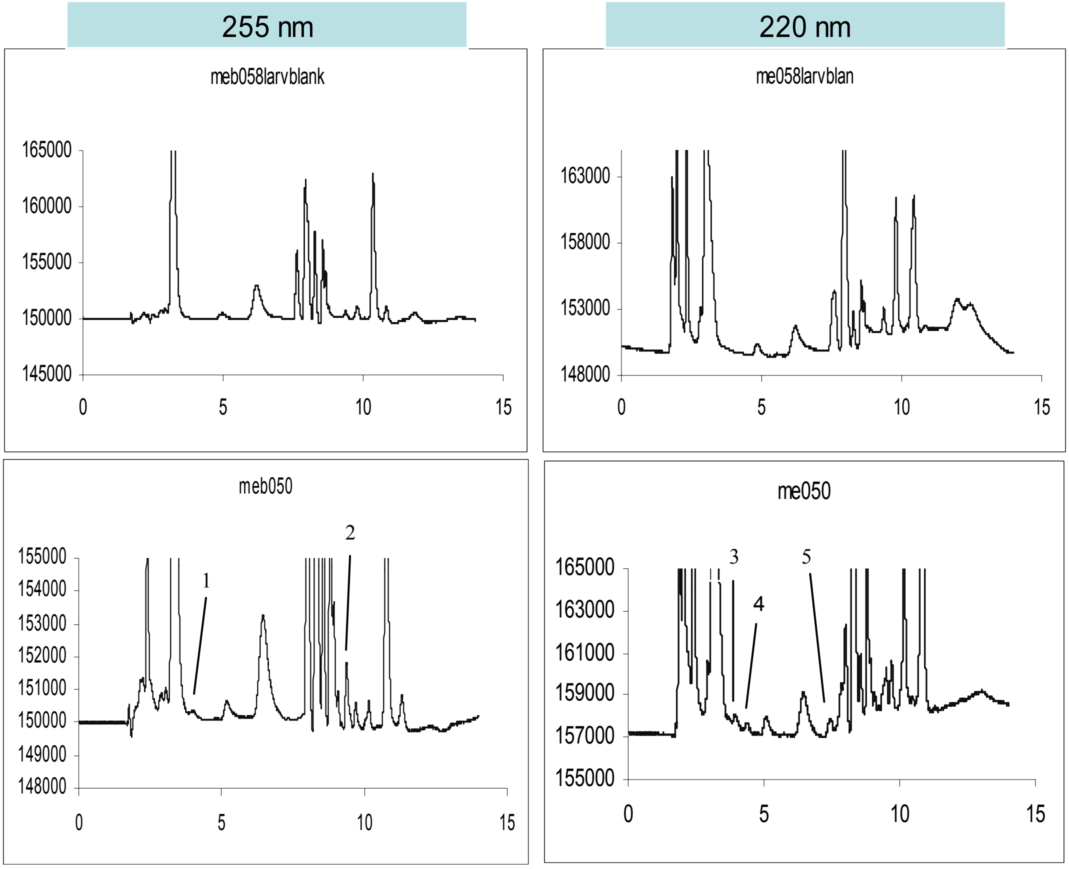


Figure 58. Typical chromatograms obtained after extraction of blank (above) and spiked (below) fish larva samples. Peak remarks are: 1: SHMET, 2: MET, 3: MTU, 4: MSO, 5: MEH

3.5. APPLICATION OF THE ANALYTICAL METHOD FOR REAL SAMPLES

According to the goals of this thesis and to the project purposes, the developed method was established for measuring real samples. The following types of samples were established:

- measuring the stability of methimazole under exposition condition
- determination of the available methimazole concentration in the exposition tanks (water)
- determination of methimazole and its metabolites from the fish whole body that were exposed to methimazole.

Expositions to the chemical(s) were carried under controlled conditions in an exposition box-room [Phytotron] (Figure 59.). Parameters were set providing optimal circumstances for the fishes. Following settings were adjusted:

Temperature: 28°C
Humidity: 45 %
Lightning: light-dark cycles in 12 hours changing (6 a.m.-6 p.m.)

Figure 59. Phytotron

Fishes were held in 17 litres tanks, tap water was used to fill tanks up. Fishes were fed by feeding automats, dry fish food were dealt out into the portion units and portioned out into the tanks twice a day (7a.m.and 5 p.m.)

3.5.1. STABILITY TEST

Standard methimazole solutions were prepared in water in three different concentrations and in three parallel dilutions for each concentration. The applied concentrations were 1, 10 and 50 ppm. Then the vials with the standard solutions were put into the Phytotron and exposed to the conditions. Sampling was carried out by automated pipettes, 500 µl exposed standard solutions were pipetted out and filtered in to 1.5 ml HPLC vials. Peak areas were measured for all samples and compared to the standard peak areas as reference. The results are presented in the figure 60 as recoveries of the reference values.

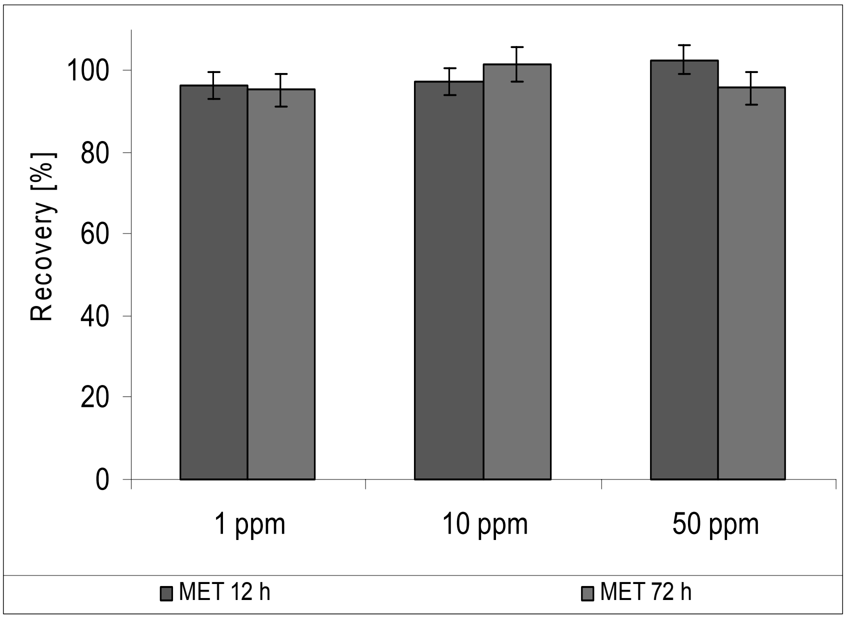


Figure 60. Methimazole stability test. Recoveries of the initial methimazole concentration after short (12h) and long (72 h) time exposition at three different levels.

As the box diagrams show, there was no degradation effect of methimazole to obtain neither after 12 nor after 72 hours, although the substance was labeled as light sensitive.

Since totally water volume exchange was planed after each 3 day periods, it could be concluded, that methimazole was not degraded and was present in the experimental period.

3.5.2. EXPOSITION AND FISH MAINTENANCE

Another application of the analytical method was the determination of the methimazole concentration both in the water of the exposition tanks and in the exposed fish whole body homogenates. Methimazole concentrations were determined each day during the exposition to control the availability of the given amount of the endocrine disruptor. After exposure fishes were homogenised and measured. Both adult and larvae fish were used for the experiments.

BREEDING OF THE FISH

Adult animals originated from the Zebrafish Laboratory of the Institute for Developing Genetic, IDG, GSF. The animals were cultivated according to Westerfield.

Fish larvae were won by pairing male and female adult fishes in to a pairing-box filled with osmosis water. Altogether 20 male and 10 female were put in to the box. The eggs were collected and pooled until further proceedings. Animals were kept in Hanks embryo medium (HM) for breeding and during the experiences. The composition of the Hanks medium was:

Table 32. Solutions for preparing Hanks medium

	Ingredient	Concentration [g/l]
Stock I	NaCl	80
	KCl	4
Stock II	Na_2HPO_4	3.58
	KH_2PO_4	6
Stock III	$CaCl_2$	14.4
Stock IV	$MgSO_4$ $7H_2O$	24.6
Stock V	Hepes	238

Hanks medium was prepared by mixing 100 ml stock I, III and IV with 10 ml stock II and 20 ml stock V. The mixture was supplemented to 100 ml with Milli-Q water.

About 100 pieces of the pooled eggs were collected into a Petri dish containing HM. Unfertilised and stayed back embryos were selected out. Petri dishes with the healthy embryos were placed into the incubator set at 28 °C until slippage (about 3-5 days after fertilisation). After slippage the embryos (about 100 pieces) were transferred into 1 litre beakers filled with 500 ml embryo medium and were placed into the phytotron. Embryos were fed twice a day after the sixth day. 300-400 embryos were transferred on the day 10 in to the tanks filled with ~ 2l embryo medium. Fishes were fed three times a day after this.
Embryos for exposition to methimazole were breed by us, the adult fishes were purchased from the IDG, GSF.

EXPOSURE: ADULTS

In scope of this experiment was to check how the gene expression of TRα, TRβ and TSH in exposed fishes changes during short and long time exposition. To be able to point it out, if it had effect (or not) on the gene expression, it was necessary to verify the availability of the endocrine disruptor both in the water (environment) used for the experiment and in the fish tissues.
Experiments with adult fishes were carried out in 17 l tanks figure 61. Adult fishes were selected by sex and both selections were divided into six groups. One control group (for both male and female tests) was formed and the others were exposed to methimazole. Methimazole concentrations were adjusted by diluting 114 g/l methimazole stock solution. The methimazole concentrations were set to 1, 5, 10, 30 and 90 ppm, respectively. Exposition time was 5, 10 and 15 days. 2x30 (30 male and 30 female) fishes were established for each concentration.

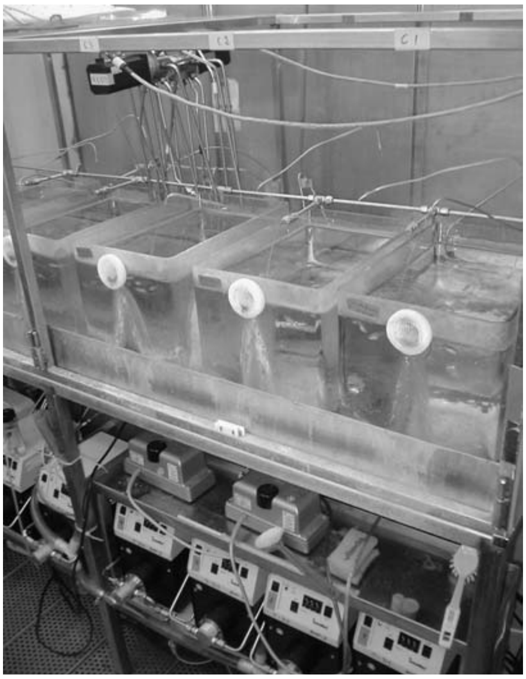


Figure 61. Adult fish exposition tanks. Below peristaltic pumps for water circulation and oxygen supplement.

5 of the 30 fishes were taken from each tank for the determination of the methimazole and its metabolites from the fish body. Sampling and sample preparation were carried out as chapter 3.4.9. described.

Water samples were taken each day within the exposure to check the available methimazole concentration. 1 ml water sample was taken from each tanks, and filtered with a Millex HS 0.2 µm filter in to 1.5 ml HPLC vials. Determination of methimazole concentration was carried out by the described HPLC method.

3.5.3. DETERMINATION OF METHIMAZOLE IN WATER

Water samples were taken from each tank on each day of the duration to determine the available methimazole amount for the fishes in it. The 15 days values are represented in the figure 62.

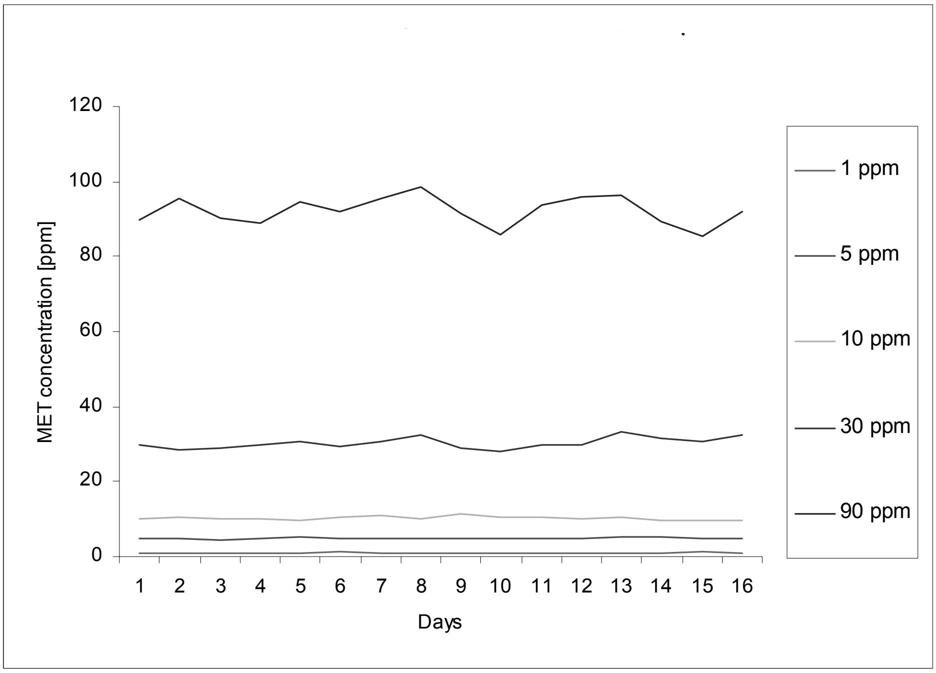


Figure 62. Methimazole concentrations in the water during the whole experiment. Samples were taken from all tanks once on each day of the duration.

Average methimazole concentration in the experimental environment was calculated for the whole experiment duration and the results are shown in the figure 63.

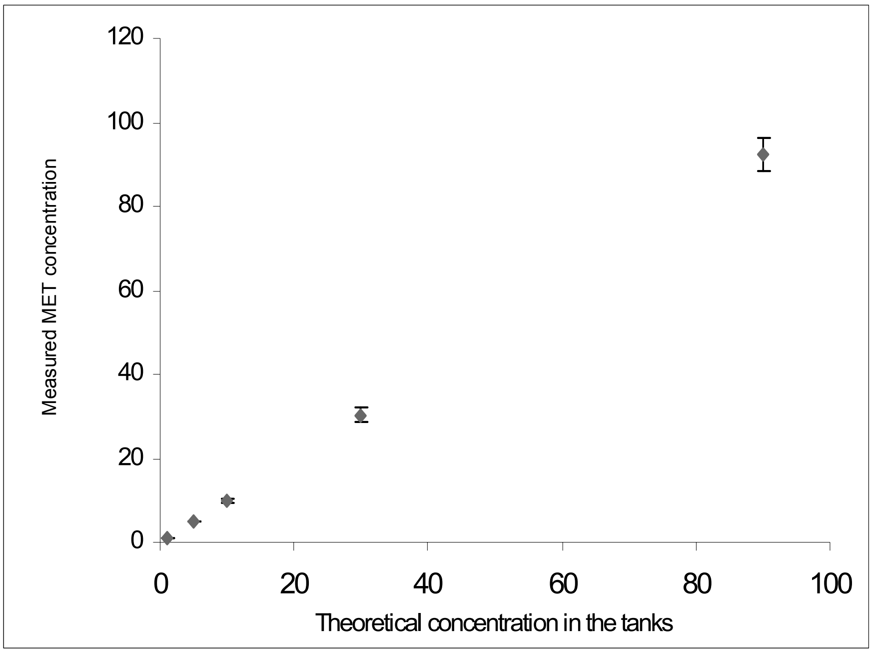


Figure 63. Average methimazole values in the exposition tanks. Average values were calculating from the daily measured concentrations during the whole experiment.

It can be seen, that the theoretical methimazole concentrations in the water corresponded well to the theoretical values. Nearly 100% of the methimazole was recovered from each tank at different concentration levels. No degradation or (a)specific adsorption of the substance were observed, which could had lead lowering the available methimazole for the fish exposition.

3.5.4. DETERMINATION OF METHIMAZOLE IN FISH SAMPLES

Five samples per tanks were taken and individually analysed. Average methimazole concentrations relating to 1g fish body weight and standard deviation values were determined for all exposition (5, 10 and 15 days). The measured values are presented in the figures 64-66.

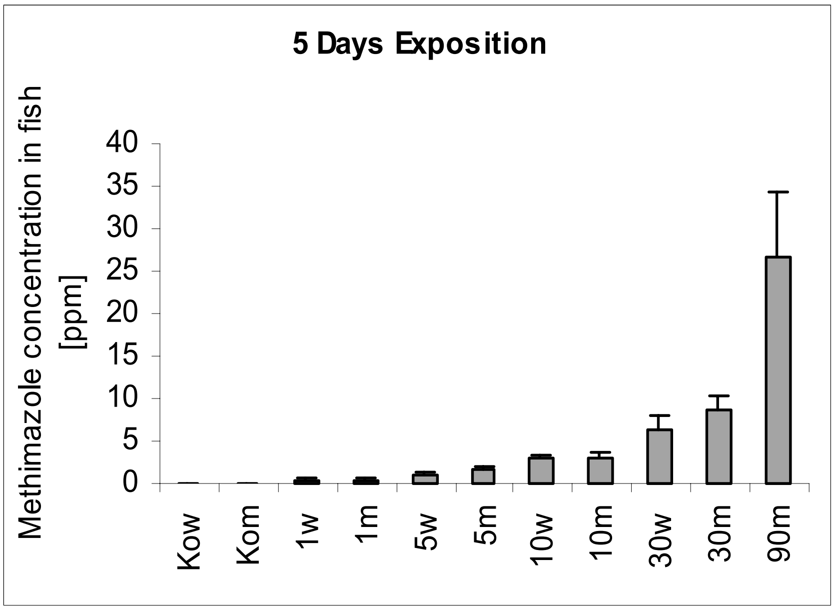


Figure 64. Methimazole concentrations in male and female fish tissues after 5 days exposition. Kow: control female; Kom: control male; 1-30w: exposed female fishes to 1-30 ppm methimazole; 1-90m: exposed male fishes to 1-90 ppm methimazole.

Even 5 days exposition showed methimazole accumulation in the fish bodies. Control groups contained no methimazole. The measured methimazole values were proportional to the values which was measurable in the water. Significant difference was verified between the different groups treated with different methimazole concentration, but significance between the male and female measured values was not verified. We defined the term bioconcentration factor (BCF) as a ratio of the test substance concentration in the fish body related to the concentration in the water (equation 15). These ratios were defined also and the results are summarised in the table 33.

$$BCF = \frac{c_{MET,fish(w/w)}}{c_{MET,water}} \qquad (15)$$

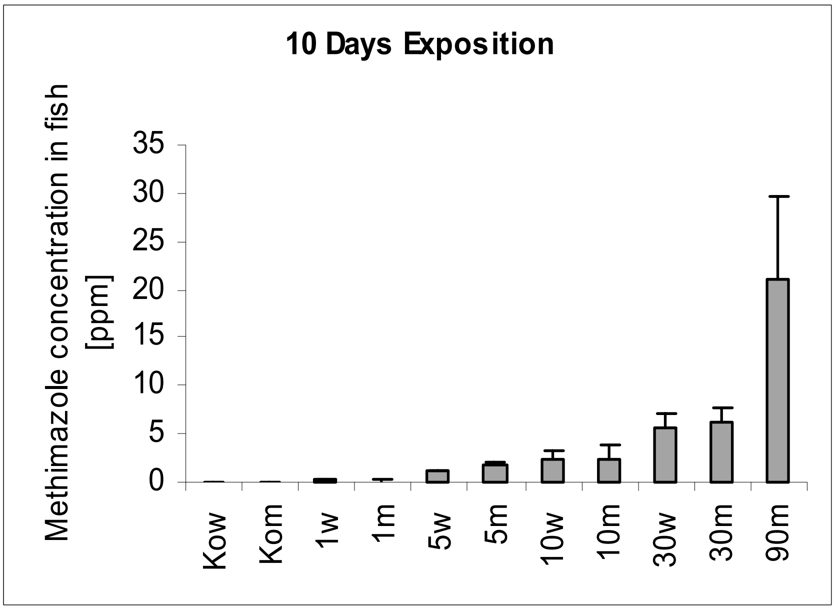


Figure 65. Methimazole concentrations in male and female fish tissues after 10 days exposition. Kow: control female; Kom: control male; 1-30w: exposed female fishes to 1-30 ppm methimazole; 1-90m: exposed male fishes to 1-90 ppm methimazole.

Even the results of the 10 days exposition verified the presence of methimazole in the exposed fishes. Measured values were significant in the case of the different concentrations but still not between the male and female samples.

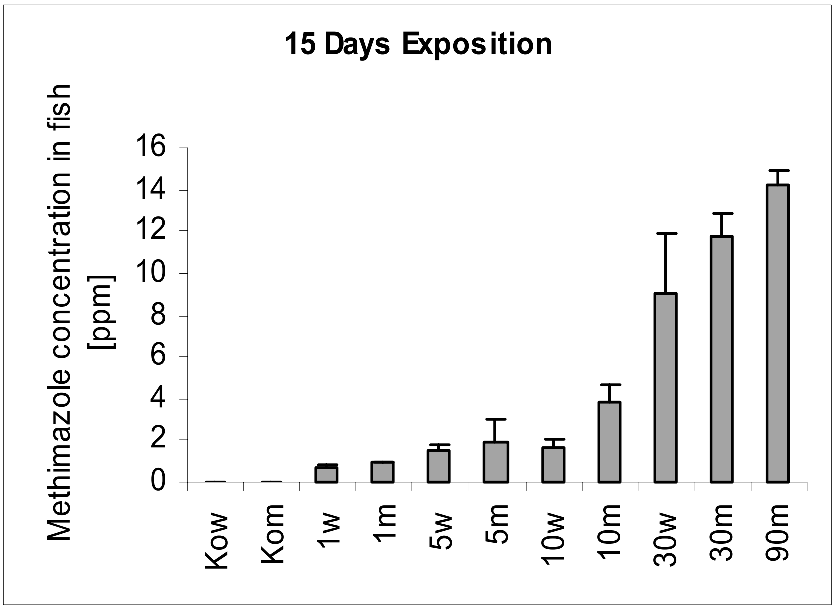


Figure 66. Methimazole concentrations in male and female fish tissues after 5 days exposition. Kow: control female; Kom: control male; 1-30w: exposed female fishes to 1-30 ppm methimazole; 1-90m: exposed male fishes to 1-90 ppm methimazole.

No methimazole was found in the control samples after 15 days exposition. Decreasing in the methimazole concentration was measured in the case of the 90 ppm treated samples related to. Significant difference between the different treated samples was verified with exception of the samples 5 and 10 ppm. Taking the expected average accumulation values into consideration, the measured low methimazole concentration of the 10w sample could have caused for this. A summary of the measured values and the calculated accumulation factors are listed below in table33.

Table 33. Average methimazole concentrations and bioconcentration factors in the exposed fish body homogenates

Sample	5 days explosion		10 days explosion		15 days explosion	
Name	MET conc. [µg/g]	BCF	MET conc. [µg/g]	BCF	MET conc. [µg/g]	BCF
Control ♂	0	-	0	-	0	-
Control ♀	0	-	0	-	0	-
1 ♀	0.42	0.42	0.17	0.17	0.68	0.68
1 ♂	0.45	0.49	0.11	0.11	0.97	0.97
5 ♀	1.04	0.20	1.13	0.22	1.47	0.29
5 ♂	1.62	0.32	1.68	0.33	1.94	0.38
10 ♀	2.85	0.28	2.40	0.24	1.67	0.16
10 ♂	3.07	0.30	2.31	0.23	3.79	0.37
30 ♀	6.18	0.20	5.62	0.18	8.98	0.29
30 ♂	8.72	0.29	6.29	0.20	11.7	0.39
90 ♂	26.6	0.29	21.11	0.23	14.2	0.16

The results of the three expositions can be summarized from analytical view as following: Different methimazole concentration for the treatment of the fishes caused significantly different concentration in the measured fish whole body homogenates. Significant difference could not be verified between the treated male and female groups at none of the same exposition levels. No significant difference was observed between the biological accumulations of the methimazole and the duration of the exposition, however a light increasing of the measured values could be observed. Opposite to that the highest exposition concentration caused significant lower bioaccumulation after longer exposition time, but the relative standard deviation value was relatively high in the case of the 5 and 10 days exposed samples. It can be stated from the results, that no longer exposition as 5 days is needed to achieve the equilibration methimazole value in the animal tissue. Longer treatment period results no higher tissue concentrations. More explanation of the results was not the task of this thesis, details can be found in related studies of the group.

METABOLITES

All samples were measured also for the metabolites of the methimazole. SHMET, MEH and MSO were not found in none of the samples, but different MTU values were measurable in some samples as it is summarised in the table 34.

Table 34. Concentration of the methimazole metabolites in the exposed fish samples

Sample code (number of samples)	Sex	Exposition (MET cc./days)	MTU conc. [µg/g fish body]	RSD [%]
44-46	M	90/15	4.65	18.4
B51-B55	M	90/10	7.87	24.6
F1-F5	M	90/5	5.82	12.9
40-43	M	30/15	2.32	32.2
36-39	F	30/15	1.52	26.2
B21-B25	F	30/10	1.87	16.4
B46-B50	M	30/10	2.45	11.2
E1w-E5w	F	30/5	0.78	8.6
E1m-E5m	M	30/5	1.21	14.6
32-35	M	10/15	0.89	22.9
27-30	F	10/15	0.93	15.3
D1m-D5m	M	10/5	1.14	23.2
22	F	5/15	0.56	-
25	M	5/15	0.32	-
26	M	5/15	0.21	-
B37	M	5/10	0.43	-
B39	M	5/10	0.65	-
B12	F	5/10	0.31	-
B14	F	5/10	0.18	-

As results show, MTU was found as only metabolite in some fish samples. Higher methimazole concentration in the water and in the tissues involved certainly measurable MTU values. Significant decrease in the measured MTU values related to the decreased methimazole levels in the water (tissue) was not observed. Relative standard deviation values were high in some cases, which are not unusual for biological samples. None of the control samples contained measurable MTU.

EXPOSURE: LARVAE

Larvae were handled as described in point breeding until processing. Exposition of the embryos was carried out in 500 ml beakers (figure 67).

Figure 67. Exposition of the zebrafish larvae

Exposition of larvae started directly after hatching and transfer step into the beakers. Duration of the exposition was 7 days, and 0 (control), 1, 5, 10 and 30 ppm concentrations were adjusted.

Exposition was carried out semi-static, the exposition medium was completely replaced every two days. Larvae were fed with conventional available dry feed twice a day during the exposition.

SAMPLING

Larvae were anaesthetised (100 mg/l Tricaine) and 100-120 larvae per concentration were collected for the determination of the methimazole and metabolites in the homogenates. Excess water was removed by Pasteur pipette, samples were weighed, washed three times with 1 ml Milli-Q water (then removed by Pateur pipette) and finally shock-frozen. Samples were stored in eppendorf vials at -80°C until homogenising.

Since it was about measuring very low amount of the test substance, even the lowest adsorbed amount of it on the fish bodies could cause false measured values. Therefore, we carried out tests by washing the samples prior to application and compared the recoverable methimazole value from larvae homogenates treated with 30 ppm methimazole after washing the samples with the unwashed control. Three parallel samples were tested. Experiment was carried out as described above. On one hand side the anaesthetised and frozen larvae were washed once or three times. The recovery values were compared with the control group recovery, which was left unwashed. The results are summarised in the table 35.

Table 35. Effect of the washing of the larva samples on the determined methimazole values in exposed samples to 30 ppm methimazole

Sample	Sample		Measured MET conc. [ppm]	Standard Deviation	BCF
	Pieces	Mass [mg]			
3x washed	100	46.8	3.86	0.65	0.12
1x washed	110	43.2	4.11	0.11	0.13
Non washed	110	46.7	5.04	0.11	0.16

It can be concluded, that washing 3x the samples reduced the measured methimazole values. Significant 22% (*p=0.05*) decreasing was observed in the measured methimazole concentration. The bioconcentration factor values were found to be in accordance with the average measurable methimazole values from the exposed adult fish samples.

According to the results above, all frozen larvae samples were washed three times with water prior to sample preparation. Larvae were homogenised by an Ultra-Turrax T25 homogenisator at 20.000 rpm, other sample pre-treatment steps were same as described in the optimised sample preparation in the chapter 3.4.9. Figure 68 represents the measured values from the exposed larva samples.

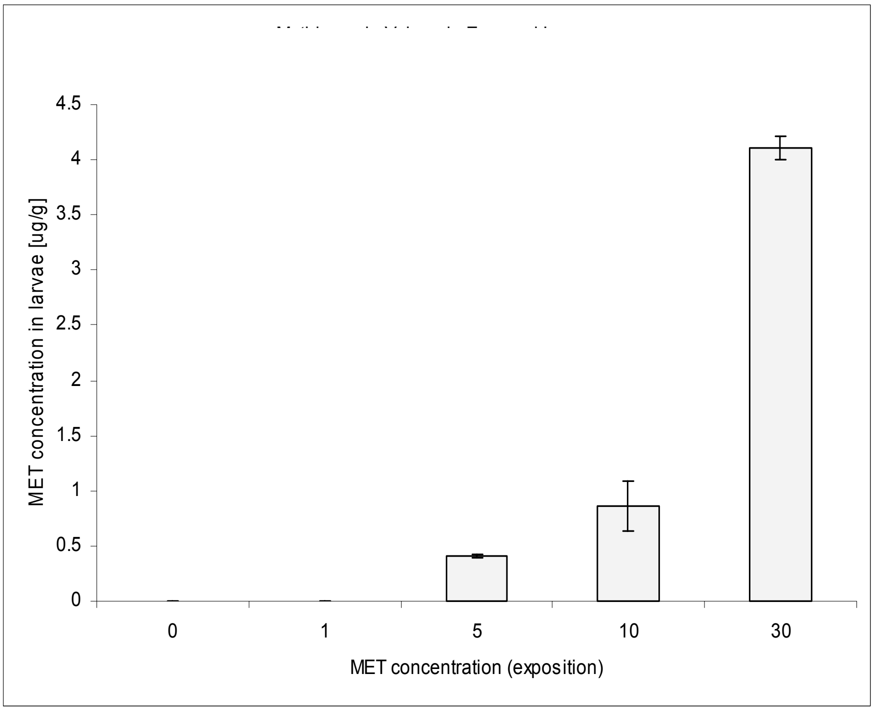


Figure 68. Methimazole concentrations in the larva tissues after 7 days exposition

As the figure shows, methimazole was found in the samples, which were exposed to 5, 10 and 30 ppm methimazole. The measured values were 0.41 for the 5 ppm exposition, 0.86 for the 10 ppm exposition and 4.11 ppm for the 30 ppm exposition, respectively. It means an about 8 % accumulation of the test substance in the larva bodies in the 5-10 ppm range. This bioconcentration factor was 0.13, that it was not accumulated in the larvae at all, since it was simple measurable from the adult samples at the same exposition level. Much rather the very low amount of the available sample (~ 50 mg) and/or the baddish treatable larvae (primarily the homogenising of the samples) can be the reason for that.

None of the metabolites were found in the embryo samples.

As summary it can be pointed out, that:

- Methimazole was detectable in the homogenised embryo tissues after exposition to that, so the biologically availability of the substance was verified for the expected provocation of the endocrine effects

- The bioconcentration factor of methimazole were found about 0.08-0.14 in the treated embryos
- MTU was as only metabolite of methimazole found in the exposed adult fish samples. No sulfoxide metabolites were found in the measured samples
- No metabolites of methimazole were found in the embryo tissue.

3.6. THYROID HORMONE ANALYSIS

Considering the low expected hormone concentrations in the zebrafish, the method requires high sensitivity and selectivity for the target analytes. Lot of parameters should be optimised to achieve the most efficient analysis. We used capillary liquid chromatographic technique for the separation and TOF mass spectrometry for the detection.
However, several separation techniques were known in the literature (chapter 2.3.) to determine thyroid hormones, the method adaptation from other equipments to the own system requires always a system specific optimisation. First of all, the eluent and the modifiers should be compatible with the mass spectrometric detector to achieve the best signal to noise ratio.

The right choice of the eluent composition, modifier, the HPLC program and tuning parameters can have influence on the sensitivity of the method. Separated peaks should be narrow and the ionisation condition should be optimal for the sensitive detection. Practical considerations were discussed in the chapter 2 according to the optimisation of the chromatographic method. In addition, Tai et al. gave a strong background for the chromatographic method development and a good overview about the problem sources. Therefore, we did not check parameters thoroughly as in the case of the method development for methimazole. The optimal conditions found for our system are summarized without demonstrating the optimisation steps.

Tuning parameters were determined by injecting 2 ppm standard thyroid hormone solutions in flow injection technique. Water, alkali buffers, their mixture with organic solvents and modifiers were tested as solvent. Sample inlet position, ion spray voltage, ionising modus, block and desolvation temperature, gas flows were optimised among others.

3.6.1. LC-MS METHOD OPTIMISATION

Capillary chromatographic technique was investigated for the measurement to achieve more narrow peaks and to increase the ionisation efficiency in the MS-ion source.

The goal of this method development was the separation and determination of the both thyroid hormone forms T3 and T4. The optimisation was carried out in relatively simply ways, since the physical-chemical properties of the two hormone forms were very similar.
The matrix components should had been considered so hard from the viewpoint of the separation, thus the MS detection could offer a selective qualification of the substances.

With taking the solubility of the target analytes into consideration, the following parameters were found useful for the separation:
Waters XTerra C18 MS 150x0.32 mm, 3 µm low bleeding capillary column (*Waters*) was used as the solid phase to reduce analysis time and solvent consuming. More details about the column was stated in chapter 3.2.1. Following mobile phase composition was found suitable for the ionisation and separation:

A: 30% acetonitrile/water
B: 100% acetonitrile containing 0.2 % formic acid

The time programming of the gradient elution is listed in the table 36.

Table 36. Gradient programming of the capLC-MS for the determination of the thyroid hormones

Time	A %	B%	Gradient
0	90	10	
2	90	10	
3	70	30	Linear
6	50	50	Linear
10	0	100	Linear
12	0	100	
12.5	90	10	linear
15	90	10	

Eluent flow rate was 8 µl/min, 1 µl sample was injected. Autosampler block temperature was set at 20°C, the column temperature at 40°C to speed up the measurement. Parallel to MS, PDA detection was applied. The detection was carried out at 240 nm.

The apolar character substances were retained satisfactory on the column. They were eluted and separated within 10 minutes. Peak shapes were good, resolution factors were acceptable (R_s~1.5), but in some cases the resolution factor values showed high relative standard deviation values in day to day relation. However, this effect was not as problematic as it would had been with conventional detectors. Typical obtained chromatograms are presented in the figure 69.

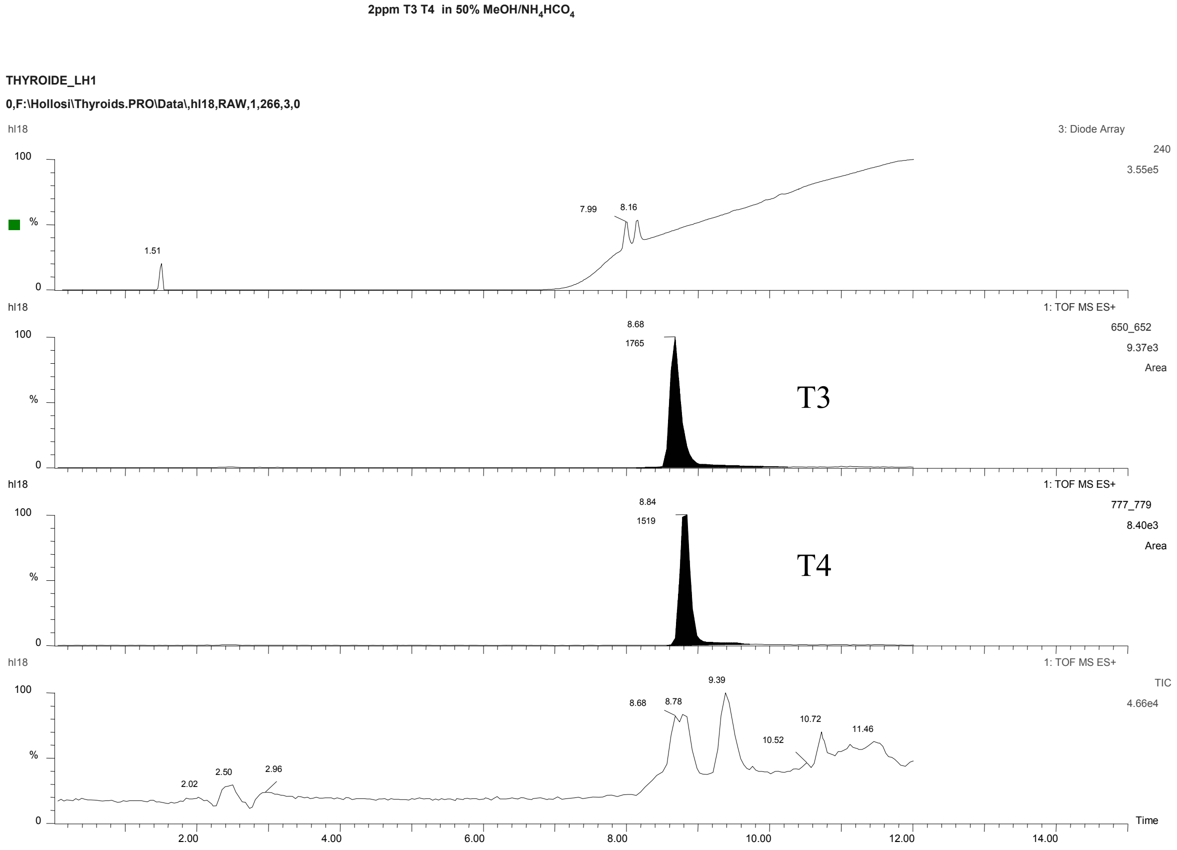


Figure 69. Typical chromatograms of a 2 ppm standard thyroid hormone mixture. MS detection was carried out in SIM mode.

OPTIMISATION OF THE MS SETTING

Following MS settings were found to be optimal for the detection.

Table 37. MS Settings for the thyroid hormone analysis

SOURCE (ESI +)	
capillary	2800 V
cone	46 V
extractor	0 V
RF lens	0.90 V
Source block temperature	80°C
Desolvation temperature	120°C
Desolvation gas flow	300 l/h
Nebulizer gas flow	50 l/h

MS2 TOF	
Transport	2 V
Aperture 2	15 V
Acceleration voltage	200 V
Focus	1 V
Tube lens	0 V
Offset 1	0 V
Offset 2	47 V
Pusher	980 V
TOF	9.2 kV
Reflectron	22.80
Time	255 µs

Typical obtained mass spectrums of a standard sample containing 20 pg T3 and T4 are presented in the figure 70. Spectra were recorded with settings listed above.

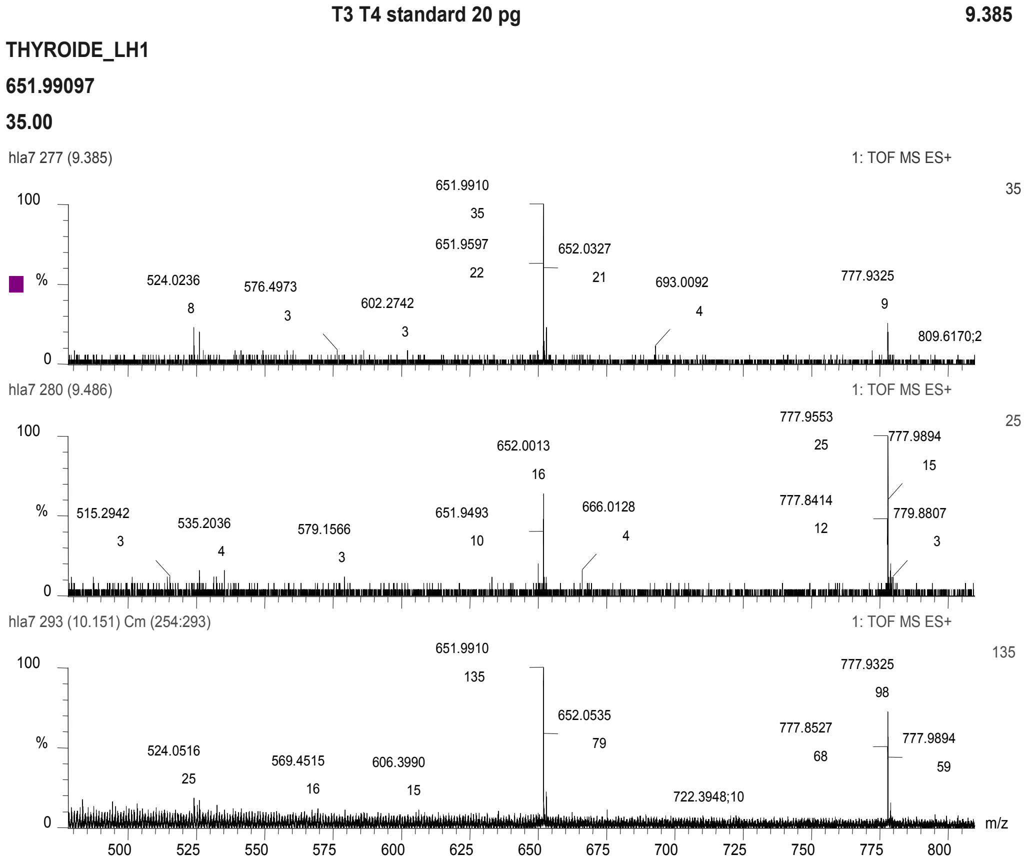


Figure 70. Mass spectra of a 20 ppb standard T3 (651.9) and T4 (777.9) mixture. Above mass spectra of the T3 and T4 peaks, below the summarized mass spectrum of the run.

3.6.1.1. VALIDATION: QUALIFICATION AND QUANTIFICATION

Validation theory and practical guide were detailed in chapter 2.9. Without repeating the aspects, we only present the results of the validation process. Exceptionally, we determined LOQ as LOQ=2*LOD.

Standard T3 and T4 solutions were prepared in 50% MeOH/ 0.05 M NH_4HCO_3. Precision of the qualification and quantification were tested with 100 ppb standard samples in five injections. Both within day and day to day precisions were determined. Accuracy was determined by adding 50 pg T3 and T4 to a 50 ppb standard solution. Peak area increasing was then compared to the theoretical value and the recovery was calculated. Results of the simplified validation are listed below, in table 38.

Table 38. Simplified validation of the thyroid hormone analysis

	Parameter	T3	T4
	t_r [min.]	7.99	8.15
Sensitivity	LOD [ppb]	2	2
	LOQ [ppb]	4	4
Precision of qualification	Within day [RSD%]	6.8 %	7.4 %
	Day to day [RSD%]	11.8%	10.2%
Precision of quantification	Within day [RSD%]	9.3 %	8.7 %
	Day to day [RSD%]	14.7 %	16.5%
Accuracy	Recovery [%]	85	82
Linearity range		1-1000 ppb	1-1000 ppb

Measured precision values were lower than 10% in the within day specifications, which are acceptable values. However, the day to day precision values were high especially in the case of the quantification, but are reasonable and lying in the range published in the literature. The apparently insufficient accuracy values are to understand with the knowledge of the high values of the precision of the quantification. Method selectivity values are in accordance with the published values.

Calibration curves were measured at seven concentrations: 1ppb, 2ppb, 10ppb, 20ppb, 50 ppb, 1 ppm and 10 ppm. Fitting of the calibration curves on the calibration points were verified by the high values of the regression coefficient. Calibration curves and its parameters are presented in the figure 71.

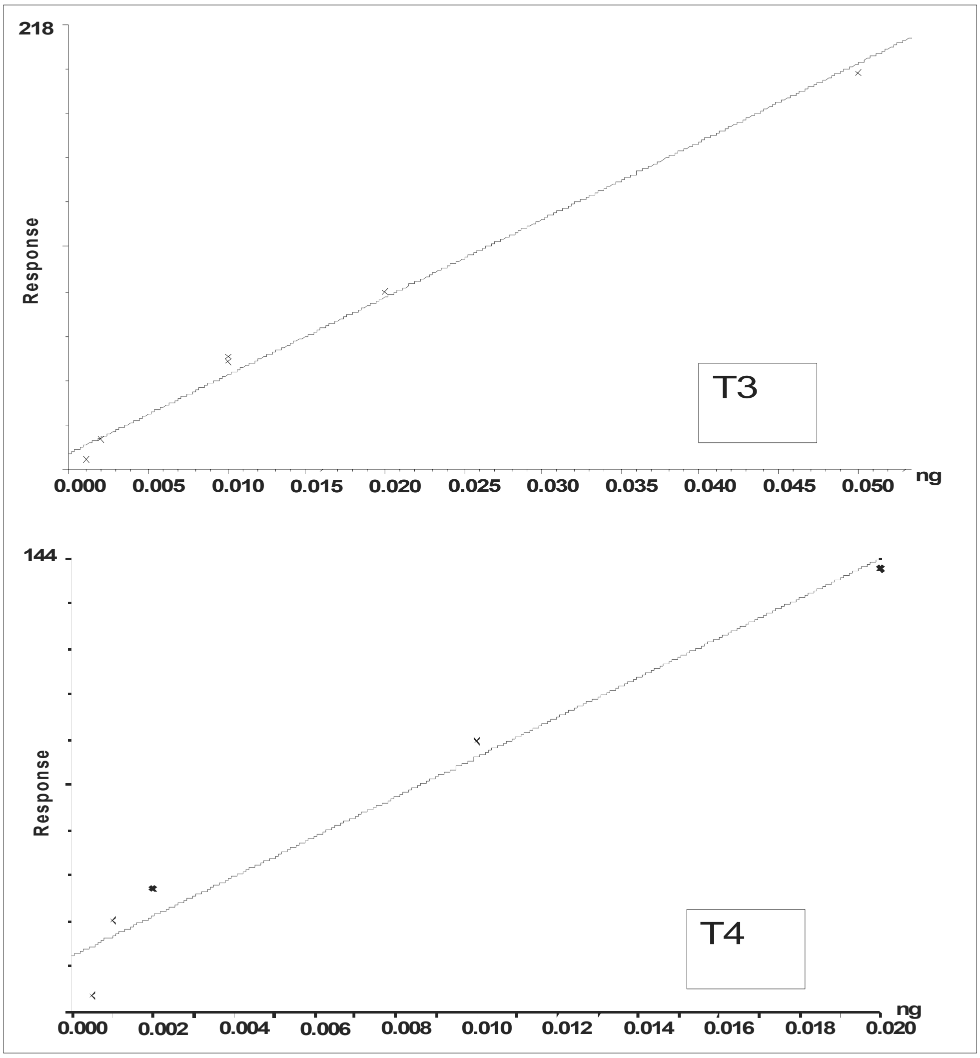


Figure 71. Calibration curves for T3 and T4 based on mass chromatograms

Calibration curve slopes were 3767.9 for T4 and 4600.6 for T3, the intercept values were 41.6 for T4 and 0 for T3. Correlation coefficients were R^2=0.9993 for T4 and R^2=0.9978 for T3, confirming the plot fitting on the measured points and so the linearity.

We can draw as short summary, that:

- The developed analytical method by capLC-TOFMS is suitable for the determination of the thyroid hormones, however the repeatability of the method is at the limit of the required range.

3.6.2. SAMPLE PREPARATION

Water solubility of the thyroid hormones is not considerable, so thus (and because of the better buffering of the system) the thyroid hormones are transported bound to carrier proteins in the living being. As mentioned in chapter 2.2., only albumin plays the key position in carrying of the thyroid hormones. Tissue concentrations of the THs were available from human medical laboratories (chapter 2). According to these sources the free (not bound) hormone reference range is 2.5-5.3 pg/ml (ppt!) for fT3 and 9-19 pg/ml for fT4, respectively.
Only a few literature data were available describing thyroid hormone concentrations in fish.
16.4-119.3 ppb T4 was measured from female rockfish samples (*Sebastes inermis)* by *Kwon et al. Carlett et al.* determined 4-45 ppt T4 and 0.8-2.8 ppt T3 from *Fundulus heteroclitus* whole body homogenate. The published values were not unified to size up the TH concentrations in zebrafish.

Because of the sensitivity of the capLC-MS equipment was not enough for the measurements in the low ppt ranges, we decided to determine total hormone values instead of the determination of the free hormone concentrations.

Injection samples by dilute and shot are manifold contraindicated. Not only the low free hormone values and the high hormone binding ratio to proteins make the analysis difficult, but also the strong matrix effect causes high ion suppression to make the analysis impossible. Therefore, a complex sample pre-treatment process is essential for the effective analytical method. The main steps of the planed preparation process are presented in the flowchart in the figure 72.

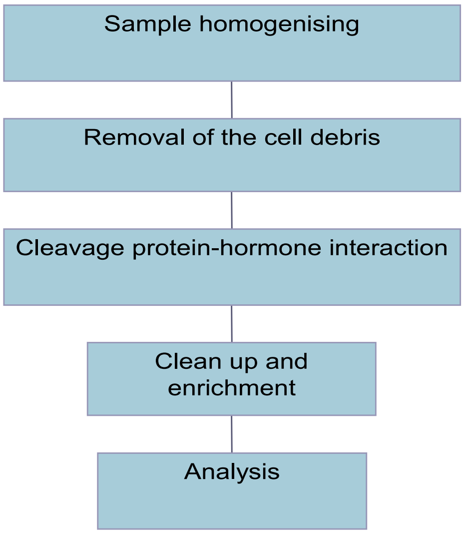


Figure 72. Main steps of the sample preparation method for thyroid hormone analysis

Sample homogenising was carried out by the Schutt-Homgen homogenator as in the case of the methimazole analysis (s. details in chapter 3.4.9.). For homogenising four times of body weight volume solvent was used. As MS tuning optimisation experiences showed, the best solubility of the thyroid hormones could be reached by using methanol/water, 50% MeOH/ 0.05 M NH_4HCO_3 or basic solvents (pH>10) for homogenising. Isoelectric point of the analytes was estimated from the pKa values calculated by the Pallas according to the relation:

$$pI = \frac{\sum_{n} pKa}{n} \qquad (16)$$

On the basis of the pI estimation of the hormones, pH~6 should be adjusted for organic extraction.

Ultracentrifuge was used for removal of the cell debris with taking into the consideration, that protein fractions may not be centrifuged out. For that goal, centrifugation was carried out only by low rpm values (~2000 x g for 10 min.) in this step.

More critical steps of the sample preparation were the cleavage of the protein-hormone bound and the enrichment of the analytes.

3.6.2.1. ON-LINE SPE

ADS COLUMN

Usage of a method is obvious, which is able to cleavage the protein-hormone interactions with simultaneous removal of the rest proteins from the solutions. The ADS column offers this opportunity, so we investigated it at first. C18 4x4 mm ADS column was used for the testing of the retention behavior of the hormones on the column. The ADS column was coupled on-line with the Perkin Elmer HPLC system. 1 ppm solution was injected to determine the retention time of the hormones. 5 and 10% MeOH/water were used as mobile phase to elute the THs from the cartridge. This eluents were selected to promote the needed conformal changes in the protein structure to set hormones free from them and were weak enough to force the free analytes retained on the C18 surface. But as good the theory sounds, so unsatisfactory results showed the practical experience: we might have repeated our experience with the methimazole and its metabolites, since we could not register any retention on the C18-diol hybrid column even in this case. The hormones were co-eluted with the also injected albumin samples (s. chapter 3.4.7.)

3.6.2.2. MULTISTEP SAMPLE PREPARATION

We tested all steps for efficiency one by one before the application of the whole process. We started with the optimization of the clean up / enrichment step. Our expectations had met with the method of Tai et al. worked out for serum samples. Both solid phase and liquid-liquid techniques were employed for cleaning and enrichment of the samples. Opposite to Tai et al. we developed a new SPE extraction method utilized the polar-apolar character of the Oasis HLB cartridges (s. details in 3.4.1.).

Chromatographic behavior of the substances suggested the adequate solid phase extraction steps. The solid phase extraction step based on the extraction of the substances from aqueous environment. We must state it out at this point, that the water solubility of the hormones is about 1.2-2 ppm. According to the human experiments and reference values it is clear that the expected total amount of the hormones does not exceed this maximal solubility value, so the

SPE extractions were carried out from aqueous medium without any modification (pH, organic solvents) to increase their solubility.

Breakthrough volume (enrichment capacity) was determined first. Taking the maximal amount of the sample volume (4 ml) into consideration, we applied 5 ml both 1 ppm and 100 ppb standard solutions on to the cartridges. We measured both concentrations in three parallel applications. Recoveries are summarized in the figure 73.

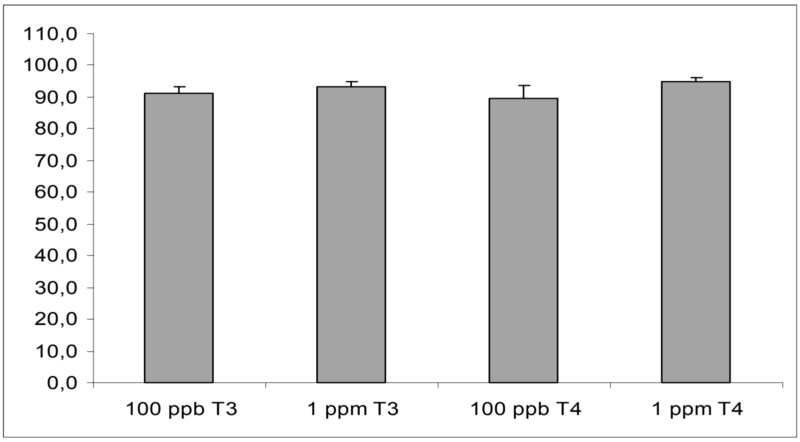


Figure 73. Retained thyroid hormone values in % on the HLB cartridge after 5 ml loading volume

As results show, thyroid hormones were retained well on the HLB cartridge even by application of higher volume than the maximal fish sample volumes.

The loaded cartridges were then washed with 2 ml water and 2 ml 50% MeOH/water solution. Finally the analytes were eluted with 90% MeOH/water from the sorbent surface of the cartridge. Average recoveries of the target substances in the collected fractions are listed in the table 39. Relative standard deviation values varied between 2-6% for all fractions.

Table 39. Thyroid hormone recoveries in the washing and eluting fractions after loading columns with 5 ml standard solutions

	T3		T4	
	100 ppb	1 ppm	100 ppb	1 ppm
Wash1	3.5%	2.1%	3.1%	2.5%
Wash2	8.5%	7.3%	7.5%	7.3%
Elution	83.7%	85.6%	84.5%	87.5%

The applied SPE method was found suitable for the enrichment of the target analytes with good efficiency on the cartridge surface. Washing with water does not elute the target substances, but 50% MeOH presence in the eluent offers enough solvent strength to elute both hormones from the column, while 90% MeOH in the eluent cause a totally removal of the substances from the cartridge. For the later applications of the method, the column was washed with 2 ml 30% MeOH/water mixture (4.5% loss for T3 and 3.8% loss for T4).

3.6.2.3. LLE

Tai et al. applied the LLE with ethylacetate for blood samples as clean up step. We checked the efficiency of the method with 1 ppm standard solution adjusted to our expectations. According to the solubility of the thyroid hormones we repeated the extraction the target analytes from the possible homogenizing solutions: both aqueous (basic) and 0-20% MeOH/water mixtures. MeOH content was limited taking into the consideration the protein solubility in organic solvents. Since keeping proteins soluble in the reaction mixture is essential to maximise the hormone extraction efficiency, maximal 20% MeOH content was tested as the limit below the proteins endure conformational changes without irreversible precipitation in the solution. 2 ml 1 ppm standard samples were prepared in 0.1 M NH_4OH and 5-20% MeOH/water and each solution was extracted with 3x2ml ethylacetate. Water (serum) extraction efficiency was described (w>80% for both) in the paper of Tai et al. The measured recovery values are represented in the figure 74.

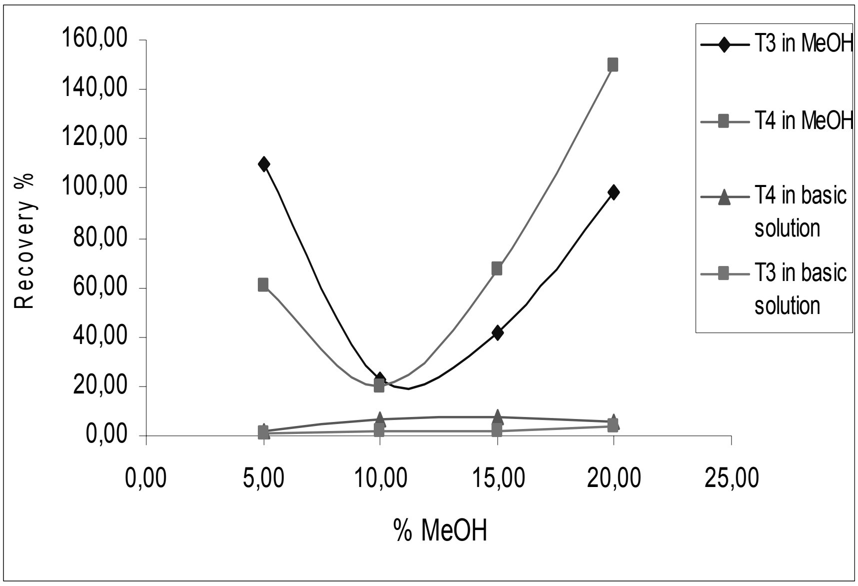


Figure 74. LLE extraction recoveries from solvents with different organic content and pH

As the plots show, liquid-liquid extractions were unsuccessful from the basic solvent and from the 10% MeOH/water mixture. Better and at the same time highly different recoveries of the T3 and T4 were observed at the 5% and 15 % MeOH/water samples. Only the extraction of the solvent containing 20 % MeOH in water showed satisfactory (98% for T3 and 145% for T4) recovery values for both substances.

Summarizing we can conclude, that the LLE method can be efficient, but only from aqueous (pH<8) or 20% MeOH/water solutions.

3.6.3. APPLICATION FOR THE MATRIX

The efficiency of the LLE and SPE methods were verified above, so the real samples were the next objects of the investigations for the method development. We first applied the following sample preparation schema based on the method by Tai et al:

Three adult fishes (0.6-1.2 g) were homogenized in 0.1 M NH_4HCO_3 containing 1 ppm both T3 and T4. Homogenates were centrifuged by 3500 x g to remove the huge amount of cell debris. Then each supernatant was acidified to pH 2 with 0.1 M HCl an equilibrated at 37°C for 2 hours. After equilibration the mixture was deproteinised with 5 ml of 150 g/l TCA in an ice bath for 30 minutes. The thyroxine was then extracted from the deproteinised sample with

5 ml of ethyl acetate (Hay et al.). The organic fraction was then evaporated under nitrogen at 40°C. Residue was dissolved in 10% MeOH/water and passed through the HLB cartridge (conditions detailed above at the method describing). After evaporation of the eluent fraction, the samples were dissolved in 50% MeOH/ 0.05 M NH_4HCO_3 for the LC-MS analysis. For testing the efficiency, the hormone concentration of the solvent used for the homogenizing was determined (100%). 10 µl samples were taken after all major steps of the sample pretreatment to determine the hormone concentrations and thereby the losses after each step. Each taken sample was evaporated and dissolved in 50% MeOH / 0.05 M NH_4HCO_3 prior to injection into the LC-MS system. Table 40 summarizes how the hormone concentration changed during the extraction:

Table 40. Thyroid hormone content changes in the samples during the different sample preparation steps. Values are recoveries of the spiked concentrations as 100%.

Step	**Recovery**	
	T3	**T4**
Before homogenising	100%	100%
Setting pH to 2	70-75%	40-55%
After deproteinisation	70-75%	40-55%
After LLE (SPE 0)	45-65%	25-34%
After SPE (**total recovery**)	**38-42%**	**23-32%**

The experiences with the method were very meaningful. The most important information can be read out from the table: ~40 % total recovery for T3 and less than 30% recovery for T4 could not be ranking among satisfactory results. Surprisingly, setting the pH to 2 caused a significant decreasing in the measurable hormone concentration. Also liquid-liquid extraction step proved not to be efficient in case of real samples with the average 20% recovery loss values. Only SPE showed good efficiency.

To check if hormone cleavage occurs during acidifying the sample, we repeated the experiment with adding DIT to the homogenizing solvent to inhibit the hormone cleavage forming DIT as end product in an equilibration process. Other conditions were the same.
We could not state any increasing in the recovery values after measuring of the fractions.

After the unsuccessfully experiments, we decided to modify and simplify the method: we tried enzymatic hydrolysis instead of acidic hydrolysis for cleavage protein-hormone interaction and liquid-liquid extraction was left out of the method.

Adult fish samples were homogenized in 0.1 M Tris puffer and centrifuged as usual. 1 ml 1.6 ppm thyroid hormone standard solution in 0.1 M Tris buffer was given to 1 ml centrifuged supernatant. Fresh prepared, 145 g/l pig trypsin solution was given to the spiked supernatant in ratio1:1. The mixture was incubated for 16 hours at 37°C incubator. The enzymatic digestion was stopped by adding MeOH (20% end concentration) to the mixture in an ice bath. After the solution was left to stay for 20 minutes, the reaction mixture was centrifuged by 80.000 x g for 40 minutes to remove proteins from the solutions. The centrifuged samples were then extracted on the HLB cartridge. Eluent was collected, evaporated and dissolved as described above. The finally measured hormone values are listed in table 41.

Table 41. Total recoveries of the thyroid hormones fro the whole process from fish samples

Substance	Total Recovery
T3	29 %
T4	31 %

Since the SPE method was found suitable for extraction of real samples with good recovery, we tried to find out, what could happen during the enzymatic digestion. Fish homogenate, 145 g/l trypsin solution and 1.6 ppm T3 and T4 standard solutions (all in 0.1 M Tris buffer) were mixed in different ratio and thyroid hormone content of the mixtures were determined after centrifugation with 80.000 x g for 40 minutes. Mixture compositions were designed to represent homogenate, standard substance and protease predominance in the solution. Each sample was evaporated and dissolved in 50% MeOH / 0.05 M NH_4HCO_3 prior to injection.
The mixture compositions are presented in the table 42.

Table 42. Solvent compositions for testing the analyte loss during the enzymatic digestion of the matrix proteins. H: homogenate, B: blank solvent

Sample number	Volume of sample (end cc %)	Volume of standard solution (end cc. %)	Volume of protease solution (end cc. %)	TH End concentration
1	1 ml H (50%)	-	1 ml (50%)	-
2	0.5 ml H (25%)	0.5 ml (25%)	1 ml (50%)	400 ppb
3	0.5 ml B (25%)	0.5 ml (25%)	1 ml (50%)	400 ppb
4	0.5 ml H (25%)	1 ml (50%)	0.5 ml (25%)	800 ppb

Further sample was spiked to 200 ppb in relation 1% end concentration of the standard, and the result was the same as in the case of the sample 1.

The measured recovery values from the samples are represented in the figure 75.

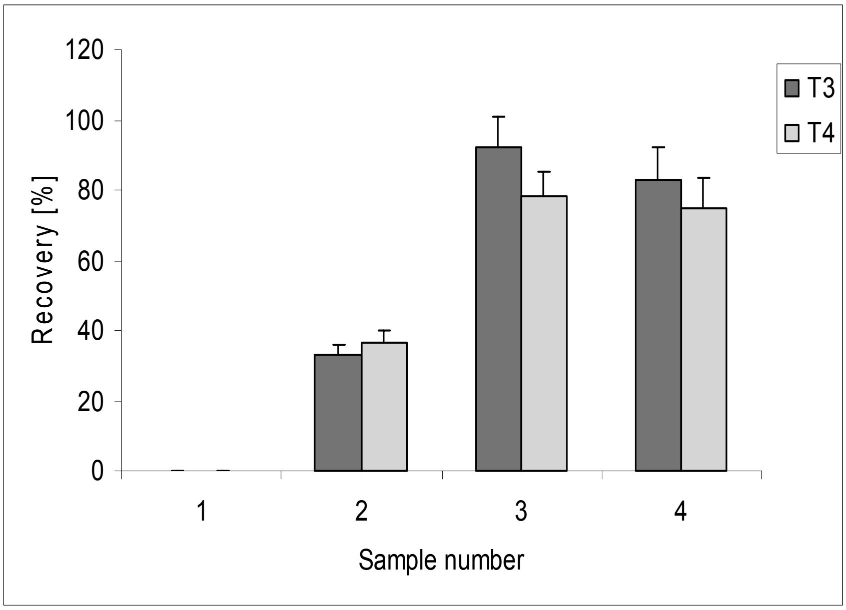


Figure 75. Thyroid hormone recoveries after enzymatic proteolysis

As the results show, there was no thyroid hormone peak measurable from the non-spiked sample 1. Acceptable recovery values were obtained in the case of blank matrix containing 25 % standard solution and in the case of standard predominance in the solution, but unfortunately none of these sample compositions reflected the real analyte/matrix ratios. However, the composition of the sample 2 correlated best with the real samples, the covered hormone values were only about 30%.

Experiences can be summarised at this point as:

- The protease solution had not been damaged the thyroid hormones, as test results showed in the blank solutions. The same results were observed when standard samples were added in excess compared to the protease quantity.
- hormone peaks got lost or only a fraction of it can be measured in the presence of the fish matrix
- no hormone peaks were detectable from the non-spiked samples, which allude partly to the imperfect protein destruction (and setting free the hormones) and partly to the high ion suppression and also matrix effect of the fish body homogenates.

In addition it can be mentioned, that because of the high ion suppression, supplementary clean up step is needed anyway.

For optimisation of the sample pre-treatment, we focused on other protein destruction methods to improve the efficiency.

OTHER METHODS TO HYDROLYSE PROTEINS

Many other methods are known for protein denaturation aside from hydrolytic methods. We investigated both physical and chemical methods to modify protein structure so, that the conjugated hormones could set free from the carrier proteins. We applied β-mercaptoethanol to reduce protein disulphide bridges, ultrasound handling and organic solvents to modify protein conformations.

3.6.3.1. ULTRASONIC BATH

Two fishes were homogenised in 0.05 M NH4HCO3 and homogenates were divided in two parts and methanol was given to the samples (5% and 15 %) to promote the conformational change. Both 5% and 15% methanol containing samples were divided again in two parts: one parts of the samples were spiked to 2.5 ppm T3 and T4 concentration, the other parts were left non-spiked.

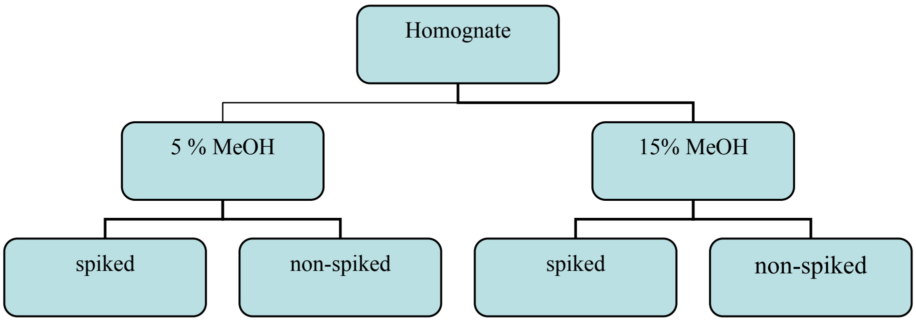


Figure 76. Schematic presentation of the experimental design for testing efficiency of ultrasonication for the cleavage of the protein-hormon interaction

All samples were (in two parallels) placed in to ultrasonic bath and sonicated for 60 minutes. Acetonitrile was added to the samples in 1:2 ratio to precipitate proteins from the mixture. After centrifugation with 80.000 x g for 40 minutes the samples were evaporated and dissolved in 50% MeOH / 0.05 M NH_4HCO_3 prior to injection. The measured hormone concentrations (recovery of the spiked values) are listed in table 43.

Table 43. Recoveries of the spiked amounts of T3 and T4after treated samples in ultrasonic bath

Samples	T3	T4
5 % non-spiked	Not detectable	Not detectable
5 % spiked	18 %	16 %
15 % non-spiked	Not detectable	Not detectable
15 % spiked	14 %	16 %

Opposite to our expectations, poor recovery values and non-detectable peaks were obtained after evaluation of the results. No peaks were found in the non-spiked 15 % MeOH samples, while T4 and T3 peaks were seen in the 5% MeOH samples, but the peak areas could not calculated because of the presence of the high number of interfering peaks. In conclusion: ultrasonic bath showed no potential to be suitable for the deconjugation. But, since the spiked recoveries are so slight, we can conceive the following:

- either the thyroid hormones will be degraded during the sonication and/
- or the hormones will be conjugated with proteins or other matrix components.

The hypothesis was not proved during the previous investigations.

3.6.3.2. EFFECT OF ORGANIC SOLVENTS AND REDUCING AGENTS

We next tried to apply organic solvents to change protein conformation and reducing agents to reduce the protein disulphide bridges both one by one and combined.

Different studies showed (ADS application catalogue), that about 4-20 % organic content in the solution can change the conformation of the proteins, while 40-45% organic content cause high efficient protein precipitation. Since our target analytes are bound to proteins, they may not be lost before deconjugation. Therefore, we built up a method on the condition that a complete deconjugation occurs before protein removal from the solution. This condition was performed by applying a gradual increasing in the organic content of the sample. During the increasing of the organic content, simultaneously increase both the solubility of the hormones in the solution and the degree of the conformational change (and even the precipitation) of the carrier proteins takes place.

Adult fishes (n=2) were homogenised in 0.1 M NH_4HCO_3 containing 5% MeOH. 4 ml homogenate was divided as follows: 2 ml of the total volume was used for the organic gradient without adding β-mercaptoethanol (O). 2 µl mercaptoethanol was added to the other 2 ml homogenate (RO). Both O and RO samples were divided in another two groups: 1 ml was taken out without spiking the samples (O and RO), while 980 µl of the parts were spiked to 100 ppb by adding 10 µl 10 ppm T3 and 10 µl 10 ppm T4 standards dissolved in 50% MeOH/ 0.1M NH_4HCO_3 to it (Osp and ROsp). After the samples were centrifuged with 3.000 x g for 5 minutes, 55 µl ACN was given to each sample. After 10 minutes incubation at 30°C, other 125 µl ACN was given to the samples and incubated for 10 minutes at 30°C. In the further step 675 µl ACN and finally 1800 µl ACN were given to the samples followed by the 10 min. incubations. The mixture was then centrifuged with 80.000 x g for 40 minutes and the supernatant was evaporated under nitrogen at 35°C. Samples were dissolved in 5% MeOH/water and were placed onto the HLB cartridges (s. details above). Elution fractions were evaporated and dissolved in 200 µl 50% MeOH/ 0.1M NH_4HCO_3 prior to measuring. Recovery and the measured hormone values are summarised in the table 44.

Table 44. Detected hormone values after treating samples with organic solvents and reducing agents (Recovery values)

Sample	T3	T4
O	100 pg	n.d.
RO	157 pg	n.d.
Osp	171 pg (34%)	163 pg (32%)
ROsp	240 pg (48%)	275 pg (55%)

As results show, T3 was measurable in both applications, while no T4 was measurable from the non-spiked samples in neither case. Spiked samples showed difference depending on the sample reducing agent content. Significant higher recovery values of the hormones were obtained in the presence of β-mercaptoethanol in the mixture. However, the recoveries were still below ~60%, the observed average 50% recovery for both substances means the best results yet.

The further investigation of the method development was based on the method above. The repeated experiments were carried out in the presence of β-mercaptoethanol as described above with the following modifications:

- fishes were homogenised in 0.05 M phosphate solution adjusted to pH 6 containing 5% ACN
- two spiking levels were tested: 200 ppb and 2 ppm (without non-spiked samples)
- parallel to the matrix samples, also blank samples were investigated at both spiking levels
- samples were incubated at 45°C for 10 minutes after spiking and before adding the first portion of acetonitrile to it.

Other parameters were as same as described above (in 3.6.3.2.). The recovery values are listed in table 45.

Table 45. Recoveries of the whole analytical method with standard and matrix samples using organic solvents for the deconjugation of the protein-hormone binding

	Spiked level: 200 ppb		Spiked level: 2 ppm	
	T3	T4	T3	T4
Blank samples (only standard)	67 %	75 %	78 %	74 %
Homogenates	38 %	43 %	51 %	54 %

However, there was no improvement observed in the recovery values, the results confirmed the recovery values measured with other samples on another day (repeatability). Significant difference was to observe between the matrix and the blank samples, which refers to a strong matrix effect of the fish body homogenates. If it is depending on a decomposition or special adsorption of the hormones in the fish matrix or the deconjugation process, is still under discussion and solving the problem needs more investigations.

Unfortunately no other investigations could be carried out in the scope of this work, so we shortly summarise the state of the things:

- LC-MS method was developed for the determination of thyroid hormones
- Application of the sample pre-treatment method for real samples was uncertain: however, clean up and enrichment methods were developed successfully, recovery rates were not better than 60% for the whole process
- insufficient deconjugation of the carrier protein-hormone interaction, strong matrix effect and decomposition of the target analytes in the matrix can be suspected to be responsible for the poor recovery values.

More details can be read about the deficiency of the sample preparation process and about the possible resolutions in the chapter summary and outlooks.

4. SUMMARY

The goal of this doctoral thesis was the developing and application of analytical methods which are suitable for the monitoring endocrine disruption test substance, and its effects on the thyroid hormone levels in zebrafish. Analytical measurement was required to approve the presence of the investigated endocrine disruptor model substance both in the exposed animal tissues and in the environment in which the test animals were maintained during the exposition. Since even the metabolites of the methimazole can influence the obtained biological effects, their presence must have been determined in the tissues also. The metabolites for analysis were selected to represent all types of main metabolising steps: oxidizing (N-methylimidazole-2-sulfinic acid, N-methylimidazole-2-sulfonic acid, N-methylhydantoin), demethylation (2-mercaptoimidazole) and bond-cleavage (N-methylthiourea).

Another goal of the thesis was the developing of an analytical method to determine the changing in the thyroid hormone levels in the exposed animals as the effect of the disruption.

Liquid chromatographic method was developed for the simultaneously determination of methimazole and its metabolites. However, there was found publications in the literature for the determination of methimazole and one of its metabolites one by one, there was no method known for the simultaneously determination of these substances within a single run. Simultaneously determination of low molecular weight, high polar substances and especially their usually more polar and more water soluble metabolites is still one of the most problematic topics of the reversed phase chromatographic practice, what was verified by our experiments also.

Various, also new developing analytical columns were tested for separation efficiency with the most important chromatographic parameters, and they were compared. Prontosil AQ Plus column proved to be limited useful, however the required minimum capacity factor was not fulfilled in the case of N-methylthiourea and 2-mercaptoimidazole. Other tested columns were unsuitable for this task.

Atlantis column fulfilled the most important chromatographic requirements and retained as required all substances of interest. Misfortunately two compounds (N-methylthiourea and 2-mercaptoimidazole) could not be separated with the required resolution, they were eluted as one peak, but depending upon the different absorbance of the substances at different UV wavelength, they could be both qualified and quantified. N-methylimidazole-2-sulfinic acid and the structurally very similar N-methylimidazole-2-sulfonic acid were not separated and so neither the qualification, nor the quantification were possible. The developed new separation method is simple, effective and enough sensitive. The new chromatographic method is capable to qualify and quantify the polar parent compound and the more polar metabolites on a reversed phase chromatographic column in one simultaneous run, which has not been published before. Simultaneous analysis of parent compound and metabolites are cost efficient and save time.

Capillary electrophoretic method was also developed to improve the separation efficiency, since the separation technique is outstanding suitable for the determination of small, high polar (ionisable) substances with high resolution and efficiency. Determination of the selected methimazole metabolites by this technique was never published before. After optimisation, the developed capillary electrophoretic method was found suitable for the separation of the methimazole and its metabolites. N-methylthiourea was found not detectable with this method. The effectivity of the mobility scale transformation was also presented and verified. With its usage, higher precision and reproducibility were achieved.

Separation parameters and efficiencies of the developed liquid chromatographic and capillary electrophoretic methods were compared. The resolution efficiency was found better in the case of the capillary electrophoretic method: N-methylimidazole-2-sulfinic acid, N-methylimidazole-2-sulfonic acid, 2-mercaptoimidazole, N-methylhydantoin and methimazole were separated with the required higher than 1.5 resolution values, while N-methylthiourea was not detectable. The chromatographic method was capable to separate only four of the six target substances (N-methylimidazole-2-sulfinic acid/N-methylimidazole-2-sulfonic acid, 2-mercaptoimidazole/N-methylthiourea, N-methylhydantoin and methimazole) however five substances (sulfoxidised metabolites, 2-mercaptoimidazole, N-methylthiourea, N-methylhydantoin and methimazole) were to quantify and to qualify. The two sulfoxidised metabolites (N-methylimidazole-2-sulfinic acid and N-methylimidazole-2-sulfonic acid) were not to differentiate, they were co-eluted.

Total analysis time was 15 minutes for both techniques liquid chromatography and capillary electrophoresis. Since capillary electrophoresis possessed higher theoretical plate numbers and so sharper peaks, the liquid chromatographic method showed lower sensitivity: lower limit of detection and so limit of quantification values.

It can be concluded that the two different separation techniques complete each other. To solve completely the analytical problem, two consecutive measurements are needed: once the samples should be measured by the developed capillary electrophoresis technique and others the samples should be measured by the developed liquid chromatographic technique to determine all components of the interest.

Adequate and attractive sample pre-treatment method was also developed and optimised, which was compatible with both developed analytical separation technique. It was difficult to find a suitable method for the simultaneously preparation of the substances. There was no selective method found for the simultaneous high efficient enrichment of the analytes, the sample pre-treatment method was better effective for the clean up process of the samples. The analytes of the interest can be selectively enriched by IEX-SPE, but only one by one (N-methylimidazole-2-sulfinic acid, N-methylimidazole-2-sulfonic acid, 2-mercaptoimidazole, N-methylhydantoin and methimazole). HLB cartridge was found suitable for a better enrichment of the main substanced methimazole.

In spite of its simplicity, the new approach (mixed polar and apolar characteristic solid phase) of the modified matrix solid phase dispersion technique applied to the fish samples showed not higher than 70% recovery, so it was found not suitable for the application.

The chosen sample preparation method by solid phase extraction (HLB) was found simple and effective for the simultaneous determination of the target analytes. Recovery values for all substances were higher than 70% from spiked fish matrix samples.

The developed analytical method was applied for measuring real samples and was found suitable. Adult fishes and larvae were exposed to methimazole and the concentration of it and its metabolites were determined. Methimazole was found in all exposed fish whole body homogenates. The measured concentrations in fish tissues correlated well with the exposition levels. Metabolites were also determined, and only N-methythiourea was found as methimazole metabolite in the zebrafish samples. The published sulfoxidised forms, which were found in human and rat, were not detectable in the tested fish samples. This was also a new establishment.

Online available freeware and commercial purchased expert systems were used for the prediction of the physical-chemical properties of the analytes of the interest. Also their efficiency was investigated. Both programmes showed problems during the prediction. Both software predicted a basic character of the imidazole based substances on the 3-nitrogene and of the N-methylthiourea, which were not experienced, as the capillary electrophoretic method also confirmed. Neither of the software recognised the modified electron structure of these compounds arisen from the presence of the 2-mercapto group.

Analytical method was also developed for the determination of the thyroid hormones from fish tissues. Capillary liquid chromatographic method with electrospray ionisation and time of flight mass analysator was applied to improve the sensitivity of the method. Unfortunately the application of the method for measuring real samples was not successful yet. Spiked analytes were not to find in the samples after preparation of the samples. We achieved only ~60 % as maximal recovery with spiked samples. But no hormone peaks were found in unspiked real samples: neither in control, nor in exposed samples. High ion suppression effect was obtained in the case of fish samples, which is not obtained with methods employing immunoassay techniques because of the different detection principles. The very complex fish matrix could be blamed for these observations. In this case further method optimisations are necessary by all means.

5. OUTLOOK

Both developed analytical methods for the determination of methimazole and its metabolites have real potential to be applied in clinical chemical laboratories for drug monitoring. Since methimazole is a widely used test substance in endocrine disruption and in other biochemical and physiological studies (N-methylthiourea also), this method will have a wide application potential in view on these fields also. In addition, the methods (due to its popularity especially the HPLC method) can serve as strong basis for further developments of determination of other low molecular weight and high polar substances. Especially other imidazole substances, derivates, structural similar forms are concerned due their important and versatile biological function. And finally but not last, even the nucleotide analysis could indirectly benefit from our results.

Thyroid hormones are routinely measured in the practice and also lot of experiments are carried out to study various effects on and of the system. It is unambiguous, that all (especially the more and more sensitive) new developments are interesting for these applications. The thyroid hormone analysis from fish samples needs further investigations to improve the efficiency. First of all, the much more effective cleavage of the hormone-protein interaction should be aimed. Any other adsorption of the hormones on the matrix components (e.g. on the flake) should be hampered (e.g. flake removing prior to analysis). The application of more selective sample preparation methods e.g. molecular imprinted polymers could improve the efficiency of the clean up and enrichment steps.

6. REFERENCES

M. **Aletrari**, P. Kanari, D. Partassides, E. Loiziu, J. Pharm. Biomed. Anal. 16(1998)785-792

P. **Batjoens**, HF de Brabander, K de Wasch, J. Chromatogr. A. 750(1996) 127-132

E.J.W. **Barrington**, N. Barron, DJ Piggins, Gen. Comp. Endocrinol. 1. (1961) 170-178

S. **Benvenga**, J. Robbins,. TEM 4. (1993) 194-198

S.T. **Bigos**, J. Maclean, B. Butler, Clin. Chem. 34 (1988) 1214

D.J. Bouma, H. Kammer, M.A. Greer, J. Clin. Endocrinol. Metab. 55(6) (1982) 1138-1142

V.I. **De Brabandere**, P. Hou, D. Stöckl, L.M. Thienpont, A.P. De Leenheer, Rapid Comm. Mass Spectr. 12 (1998) 1099-1103

P. **Broto**, G. Moreau, C. Vandycke, Eur.J.Med. Chem. – Chim. Ther. 19, 71 (1984)
D.D. Brown, Proc. Natl. Acad. Sci. USA 94 (1997)13011-13016

J. **Buhlert**, Dtsch. Lebensm. Rundsch. 82 (1986) 146

K.L. **Busch**, G.L. Glish, S.A. McLuckey, Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry, VCH, New York, 1988

E. **Campi**, G. Ostacoli, A. Vanni, Gazz. Chim. Ital. 95(1965) 796

M.A. **Carletta**, P. Weis, J.S. Weis, Marine Environm. Res. 54 (2002) 601-604

Y.F. **Cheng**, D.J. Phillas, E. Neve, L. Bean, J. Liq. Chrom. Rel. Technol. 20 (1997) 2461

D.S**. Cooper**, Am J Med 80:6(1986)1165-1168.

D.S. **Cooper**, N. Engl. J. Med. 311:21 (1984)1353-62.

E. **Cruz**, M.R. Euerby, C.M. Johnson, C.A. Hackett, Chromatographia 44/3-4 (1997) 151-161

DIN 32645 [Deutches Institut für Normung e.V. (DIN), DIN 32645-Chemische Analytic-Nachweis-, Erfassungs- und Bestimmungsgrenze. Beuth Veral, Berlin, (1994).

A. **Dicoria**, S. Morchese, R. Samperi, J. Chromatogr. B. 642(1993)309

C.G. **Duarte**, A.C.M. Polizello, A.I. Assis-Pandochi, A.C.C. Spadaro, J. Pharm. Biomed. Anal. 23(2000)237-241

J.S. **Fritz**, Analytical Solid Phase Extraction, Wiley-VCH, 1999, pp. 63-83

A.K. **Ghose**, G.M. Crippen, *J. Computat. Chem* 7, 565 (1986)

T. **Ghous**, A. Thownshend, Anal. Chim. Acta 411 (2000) 45-49

H.G. **Gika**, VF Samanidou, IN Papadoyannis, J. Chromatogr. B. 800(2004)193-201

Gilman et al editors. Goodman and Gilman"s the pharmacological basis of therapeutics, 8th edition New York: MacMillan Publishing Company, 1990: 1373-7.

J.J**. Gilroy**, J.W. Dolan, P.W. Carr, L.R. Snyder, J. Chromatogr. A 1026 (2004) 77)

E.G. **Grau**, Thyroid hormones. In “Vertebrate Endocrinology: Fundamentals and Biomedical Implications”Vol. 2, pp. 85-102, Academic Press, San Diego, 1987

A. **Hashash**, D. Lynn Kirkpatrick, M.J. Egorin, L.H. Black, J.S. Lazo, J. Chromatogr. B. 768(2002)239-246

A.L. **Hantson**, M. De Meyer, N. Guérit, J. Chromatogr. B. 807(2004)185-192

M.C. **Hennion**, J.Chromatogr. A. 885(2000) 73

H. **Hooijernik**, W.G. de Ruig, J. Chromatogr. 394 (1987) 403-407)

K. **Ienaga**, K. Nakamura, F. Naka, T. Goto, Biochem. Biophys. Acta 967(1988)441-443

J.V. **Iribarne**, B.A. Thompson, J. Chem. Phys. 64(1976) 2287-2294

R. **Jansson**, J. Clin. Endocrinol. Metab. 57:1(1983)129-32.

R. **Jansson**, B. Lindstrom, P.A. Dahlberg, Pharmacokinetic properties and bioavailability of methimazole. Clin. Pharmacokinet. 10 (1985) 443-450

M.C. **Jennion**, J. Chromatogr. A. 856(1999)3

R.J. **Kavlock**, G.P. Daston, C. DeRosa, P. Fenner-Crisp, L.E. Gray, S. Kaatari, G. Lucier, L. Luster, M.J. Mac, C. Macza, R. Miller, J. Moore, R. Rolland, G. Scott, D.M. Sheedan, T. Sinks, H.A. Tilson, Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S.-EPA-sponsored workshop. Environ. Health. Persp. 104, Suppl. 4. (1996) 715-740.

J.P. **Kampmann**, Hansen JM., Clin. Pharmacokinet. 6 (1981) 401-428

A.G. **Kazemifard**, D.E. Moore, A. Aghazadeh, J. Pharm. Biomed. Anal. 25 (2001) 697-711

K. **Kimota**, K. Iwaguchi, S. Omishi, K. Jinno, R. Eksteen, K. Hosoya, M. Araki, N. Tanaka, J. Chrom. Sci. 27(1989 Dec.) 721-728

H. **Kobayashi**, O. Matano, S. Goto, J. Chromatogr. 207(1981) 281-285

N. **Kobayashi**, M. Kadena, S. Teramoto, Tox. Letters 12 (1982) 109-113

S. **Kontou**, D. Tsipi, V. Oreopoulou, C. Tzia, J. Agric. Food. Chem. 49(2001)1090-1097

R.J. **Krause**, S.C. Glocke, A.A. Elfarra, Drug Metab. Dispos. 30:10 (2002) 1137-1142

A.V. **Kudryavtsev**, I.V. Perminova, V.S. Petrosyan, *Analytica Chimica Acta* 407 (2000) 193.

J.Y. **Kwon**, Y.J. Chang, Y.C. Sohn, K, Aida, J. Fish. Biol. 54. (1994) 370-379

P.A. **Langhorne**, ADIS Press International, Inc., 1989.

P. **Laurberg**, Acta Endocrinol. 111(1986)39-43.

P. **Laurberg**, J. Torring, J. Weeke, Acta Endocrin; 108(1985)51-54.

J.F. **Lawrence**, F. Iverson, H.B. Hanekamp, P. Bos, R.W. Frei, J. Chromatogr. A. 212(1981) 245-250

J.F. **Lees**, W.D. Alexander, M. Lewis, D.C. Evered, Endocrin. 100:3 (1977) 765-769

P. **Licht**, M.F. Moore, Arch. Biochem. Biophys. 309 (1994) 47-51

C.E. **Lin**, W.C. Lin, W.C. Chiou, J. Chromatogr. A, 705 (1995) 325

Y.-W. **Liu**, W.-K. Chan, Differentiation 70(2002)36-45

H.M. **Lott**, S.A. Barker, J. AOAC Int. 76/3(1993)663-668

L.J. **MacDonald**, N.I. Robin, L. Siegel, Clin. Chem. 24 (1978) 652

R.E. **Majors**, LC-GC Int. 4(1991)10

G.J. **Mammen**, editor. Clinical pharmacokinetics drug data handbook 1989. Langhorne, PA: ADIS Press International, Inc., 1989.

E. **Matisova**, S. Skrabakova, J. Chromatogr. A 707(1995)145

A.J.**Matty**, Fish endocrinology, Croom Helm, London, 1985

I.R. **McDougall**, J. Clin. Pharmacol. 1981 Aug–Sep; 21: 365-84

R.R. **McDowall**, J. Chromatogr. 492(1989)3

F.W. **McLafferty** (Ed.) Tandem Mass Spectrometry, Wiley, New York, 1983

B. **Michalke**, P. Schramel, H. Witte, Biol. Trace Elem. Res. 78 (2000) 67-79

B. **Michalke**, P. Schramel, H. Witte, Biol. Trace Elem. Res. 78 (2000) 81-91

R.R. **Miller**, D.J. Greenblatt (editors), Handbook of drug therapy. New York: Elsevier, 1979: 911-918

T. **Mizutani**, M. Murakami, M. Shirai, M. Tanaka, K. Nakanishi, J. Appl. Toxicol. 19:3 (1999) 193-198

T. **Mizutani**, K. Yoshida, M. Murakami, M. Shirai, S. Kawazoe, Chem. Res. Toxicol. 13:3(2000) 170-176

P. **Moree-Testa**, Y. Saint-Jalen, A. Testa, J. Chromatogr. 290(1984)263-274

G. **Moretti**, P. Betto, P. Cammarata, F. Fracassi, M. Giambenedetti, J. Chromatogr. 616(1993)291-296

B.E.P. **Murphy**, C.J. **Pattee**, J. Clin. Endocrinol. 24 (1964) 187

N. **Nabil**, D.J. Miner, J.M. Amatruda, J Clin Endocrinol Metab 54:1(1982)180-181.

N. **Nagae**, T. Enami, “Development of a new triacontyl (C30) bonded silica for high performance liquid chromatography: application of high throughput separation and sample enrichment” paper 1398P, presented at the 53rd Pittsburgh Conference on Analytical Chemistry and Applied spectroscopy, New Orleans, Louisiana, USA, 17-22 March, 2002

N.**Nagae**, U.S. Patent Nr. 6,241,891, 5 June 2001

D.O. **Norris**, Trans. Am. Fish. Soc. 98 (1969) 104-106

P. **Nuutila**, P. Koskinen, K. Irjala, Clin. Chem. 36 (1990) 1355

K. **Okamura**, H. Ikenoue, A. Shiroozu ,K. Sato, M. Yoskinari, M. Fujishima, J. Clin. Endocrinol. Metab., 65:4 (1987) 719-23.

A. **Okune**, Acta Endocrinol., 115 (1987)112-8.

M.F. **Pochard**, M. Karageorgis, M. Chavalier, J. Chromatogr. 298(1984)183-188

B.A. **Petersen**, R.N. Hanson, R.W. Giese, J. Chromatogr. 126 (1976) 503

V. **Pichon**, C. Cau Dit Coumes, L.Chen, S. Guenu, MC Hennion, J. Chromatogr. A 737(1996)25

W. **Pipkin**, Am. Lab. Nov. 1990 40D

C. **Polson**, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, J. Chromatogr. B. 785(2003)263-275)

Product Information: Tapazole®, methimazole. Jones Pharma Inc., St. Louis, Missouri (PI revised 07/14/1999) PI reviewed 06/2001.

M. **Przybyciel**, M.A.Santangelo, “ A unique hydrocarbon based stationary phase for HPLC with highly aqueous mobile phases” paper presented at the 49th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, New Orleans, Louisiana, USA, 1-6 March 1998

R.F. **Rekker**, De Kort, H.M., *Eur. J. Med. Chem.* 14 (1979) 479

S.J. **Richardson**, A.J. Bradley, W. Duan, R.E.H. Wettenhall, P.J. Harms, J.J. Babon, B.R. Southwell, S. Nicol, Am. J. Physiol. 266. (1994) R1359-R1360

T. **Roti**, Am. Int. Med. 111:2 (1989)181-182

J.H. **Romaldini** , J Clin Endocrinol Metab 57:3 (1983) 563-570.

M. **Sanchez-Carbayo**, M. Mauri, R. Alfayete, C. Miralles, F. Soria, Clin. Biochem. 32 (1999) 395-403

C. **Sanchez-Pedreno**, MI Albero, MS Garcia, V Rodenas, Anal. Chim. Acta 308(1995)457-461

E.T. de los **Santos**, E.L. Mazzaferri, Postgrad. Med.1990; 87(5): 277-278, 281-286, 291-294.

J. **Sherma**, J. AOAC Int. 80:2 (1997) 283-287

R. **Schilt**, J.M. Weseman, H. Hooijerink, H.J. Korbee, W.A. Traag, M.J. van Steenbergen, W. Haasnoot, J. Chromatogr. 81(1989)127

C. **Schmeizeisen-Redeker**, L. Bueftering, F.W. Roellgen, Int. J. Mass Spectrom. Ion Processes 90(1989) 139-150

L. **Schmidt**, J.S. Fritz, J. Chromatogr. 640(1993) 145

L.W. **Scmidt**, Ph.D. thesis, IOWA State University, Ames, IA, 1993, p. 50

S.C. **Smith**, M.G: Khaledi, Anal. Chem., 65 (1993) 193

Ph. **Schmitt-Kopplin**, A.V. Garmash, A.V. Kudryavtsev, F. Menzinger, I.V. Perminova, N. Hertkorn, D. Freitag, V.S. Petrosyan, A. Kettrup, *Electrophoresis* 22 (2001) 78

G. **Schreiber**, S.J. Richardson, Biochem. Physiol. 116 (1997) 137-160

L. **Siekmann**, Biomed. Environm. Mass Spectr. 14 (1987) 683-688

D.S. **Sitar**, Metabolism of thioamide antithyroid drugs. Drug Metab Rev 1990; 22(5): 477-502

G.G. **Skellern**, Br. J Clin. Pharmacol. 9 (1980) 137-143

L.R. **Snyder**, J.W. Dolan, J. Chromatogr. A 892:1-2 (2000)107-121

L.R. **Snyder**, J.W. Dolan, P.W. Carr, J. Chromatogr. A 1060 (2004)77-116

N. **Soukhova**, O.P. Soldin, S.J. Soldin, Clin. Chem. Acta 343 (2004) 185-190

Sparc internet site: **http://ibmlc2.chem.uga.edu/sparc**

B. **Stanovnik**, M. Tisler, Anal. Biochem. 9(1964)68

S.S.C. **Tai**, L.T. Sniegoski, M.J. Welch, Clin. Chem. 48:4 (2002) 637-642

M. **Takahashi**, M. Nagashima, S. Shigeoka, H. Kamimura, K. Kamata, J. Chromatogr. A. 958 (2002) 299-303

Tapazole package insert (Lilly—US), Rev 12/4/89, Rec 5/16/90

A. **Taurog**, M.L. Dorris, F.S. Gauziec, Endocrin. 124:1 (1989) 30-39

T. **Tatsuhara**, F. Tabuchi, M. Unate, J. Chromatogr. 339(1985)149-156

B. **Velkeniers**, R. Cytryn, L. Vanhaelst, M.H. Jonckheer, Lancet 1988 May; 21: 1127-1129.

E. **Viana**, J.C. Molto, A. Fouty, J. Chrom. A. 754(199) 437-444

L. **Vittozzi**, G. De Angelis, Aquatic Toxicol. 19:3 (1991)167-204

M. **Vogeser**, Clin. Chem. Lab.Med. 41(2) (2003) 117-126

H. **Yu**, J. Immun. Methods 218 (1998) 1-8

D. **Wagrowski-Diehl,** “development of new HPLC columns for the retention and separation of highly polar compounds” paper number 133, presented at the 53rd Pittsburgh Conference on Analytical Chemistry and Applied spectroscopy, New Orleans, Louisiana, USA, 17-22 March, 2002

M.J.M. **Wells**, General Proceedings for the Development of adsorption trapping methods used in herbicide residue analysis, in Proceedings of the 2nd Annual International Symposium on Sample Preparation and Isolation Using Bonded Silicas, Analytichem Int., Harbor City, CA, 1985, pp. 63-68

Waters SPE Product Catalogue, 2002

A **Whang**, L Zhang, Y Fang, J. Pharm. Biomed. Anal. 23 (2000) 429-436

N.S. **Wilson**, M.D.Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171

P.L. **Zhu**, J.W. Dolan, L.R. Snyder, j. Chromatogr. A. 756:1-2 (1996) 41-50

6. APPENDIX

I. LIST OF TABLES (PAGE)

II. LIST OF FIGURES (PAGE)

III. CHEMICALS

- 1-methylhydantoin, 97%, Ord. Nr.: M49887, Sigma-Aldrich Chemie GmbH, Munich, Germany
- 2-Mercaptoethanol, 99%, Ord. Nr.: 1.15433.0100, Merck, Darmstadt, Germany
- 2-mercaptoimidazole, 98%, Ord. Nr.: 284297, Sigma-Aldrich Chemie GmbH, Munich, Germany
- 3,3′,5-Triiodo-L-thyronine, 95%, Ord. Nr.: 56,460-5, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Acetonitrile, gradient grade for HPLC, Ord. Nr.: 34851, Riedel de Haen, Sigma-Aldrich, Seelze, Germany
- Albumin solution, bovine serum Ord. Nr.: A0336, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Ammonium bicarbonate, purum p.a., 99.0% Ord. Nr.: 09832, Fluka, Buchs SG, Switzerland,
- Ammonium hydroxide, puriss. p.a., 25% NH_3 in water, Ord. Nr.: 09860, Fluka, Buchs SG, Switzerland,
- Benzylamin, purum 98% Ord. Nr.: 13190, Fluka, Buchs SG, Switzerland
- BIORAD RC DC Protein Assay Kit, Biorad Laboratories GmbH, Munich, Germany
- Buthylbenzole, 99% Ord. Nr.: B9,020-3, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Dichloromethane, Pestanal, Riedel de Haen, Sigma-Aldrich, Seelze, Munich, Germany
- Disodium hydrogen phosphate, ACS reagent 99%, Ord. Nr.: S9763, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Ethylacetate, ACS reagent Ord. Nr.: 319902, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Florisil, heat activated, 100-200 mesh, Ord. Nr.: 22,073-6, Promochem, Wesel, Germany
- Formic acid, 100%, Ord. Nr.: 33015, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Hydrogen chloride, 32%, Merck, Darmstadt, Germany
- Hydrogen peroxide, Perhydrol 30%, Ord. Nr.: 7298.0250, Merck, Darmstadt, Germany
- Methanol, LiChrosolv HPLC gradient grade, Ord. Nr.: 1.06009.2500, Merck, Darmstadt, Germany
- Methimazole, Ord. Nr.: M460-0, Sigma-Aldrich Chemie GmbH, Munich, Germany
- N-methylthiourea, Ord. Nr.: M0520, Sigma-Aldrich Chemie GmbH, Munich, Germany

- Penthylbenzol, 99% Ord. Nr.: 11.317-4, Sigma-Aldrich Chemie GmbH, Munich, Germany
- o-Phosphoric acid, min. 85% p.a. Merck, Darmstadt, Germany
- Sodiumhydroxide, reagent grade 98% pellets, anhydrous, Ord. Nr.: S5881, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Sodiumdihydrogenephosphate monohydrate, Ord. Nr.: 1.03090.0250, Merck, Darmstadt, Germany
- Technical buffers pH 4, 7 and 10 for calibration, Ord. Nr.: 10880400.02, WTW
- O-Terphenyl, 99% Ord. Nr.: T280-0, Sigma-Aldrich Chemie GmbH, Munich, Germany
- L-Thyroxine, 98% Ord. Nr.: T2376, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Trichloroacetic acid, ACS reagent, Ord. Nr.: T6399, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Triethylamine, 99%, Ord. Nr.: 808352, Merck, Darmstadt, Germany
- Triphenylene, 98% Ord. Nr.: T8,260-0, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Tris(hydroxymethyl)-aminomethan, Ord. Nr.: 8382, Merck, Darmstadt, Germany
- Trypsin, from hog pancreas [E.C. 3.4.21.4.] Ord. Nr.: 93615, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Zinc-sulphate monohydrate, Ord. Nr.: 56495, Fluka, Buchs SG, Switzerland,

IV. EQUIPMENTS AND MATERIALS

- HPLC pump: PE Series 200 pump, Perkin Elmer Inc., USA
- Autosampler: PE ISS 200, Perkin Elmer Inc., USA
- Diode Array Detector: PE 235 C, Perkin Elmer Inc., USA
- System control and data handling: Turbochrom3, Perkin Elmer Inc., USA
- Capillary Chromatography system: Waters capLC, Waters GmbH, Eschborn, Germany
- Mass spectrometer: Micromass Q-TOF-2^{TM}, Waters GmbH, Eschborn, Germany
- UV-VIS Spectrofotometer: Ultrospec 3300 Pro, Amersham Biosciences Europe GmbH, Munich, Germany
- Homogenisator: Schütt-Homgen Plus, Neolab Migge GmbH, Heidelberg, Germany
- Cooler: Lauda K2R, Neolab Migge GmbH, Heidelberg, Germany
- Ultra-Turrax T25, Janke & Kunkel, Neolab Migge GmbH, Heidelberg, Germany
- Millex HS 0.2 µm syringe filter (2.7 cm), Millipore GmbH, Schwalbach, Germany
- Milli-Q water system, Millipore GmbH, Schwalbach, Germany
- Sartorius R 200 D balance, Sartorius AG, Goettingen, Germany
- Sartorius 3716 MP balance, Sartorius AG, Goettingen, Germany
- Sartorius BA210S and ME215S, Sartorius AG, Goettingen, Germany
- Vacuum SPE system, Supelco, Sigma-Aldrich Chemie GmbH, Munich, Germany
- Beckman L-60 Ultracentrifuge, Beckman-Coulter GmbH, Krefeld, Germany
- capillary electrophoresis: Beckman P/ACE 5510 and P/ACE 2100 coupled with on-line UV and DAD detection
- capillaries: -Polymicro, conventional silica tube, 50 µm and 75 µm inner diameter, Product Nr.: TSP050375 and TSP075375
- magnetic stirrer: Roth, R1000, Neolab Migge GmbH, Heidelberg, Germany
- pH-meter: Sartorius, Professional Meter PP-20, Sartorius AG, Goettingen, Germany
- pipettes: Gilson 10-100 µl and Gilson 100-1000 µl, Migge GmbH, Heidelberg, Germany
- Cameo nylon 0.2 µm (1.8 cm), Sigma-Aldrich Chemie GmbH, Munich, Germany
- syringes: Hamilton 50 µl and 250 µl pipettes, Migge GmbH, Heidelberg, Germany
- beakers, glass pipettes, glass vial holders, graduated flasks, water-jet vacuum pump for filtration glass device.

V. PHYSICAL PROPERTIES OF THE TEST SUBSTANCES

Name:	1-methyl-2-mercapto-imidazole	2-mercapto-imidazole	N-methylthiourea	N-methyl-hydantoine	3,3′,5-Triiodo-L-thyronine	3,3',5,5'-tetraiodo-L-thyronine
Abbreviation:	MET	SHMET	MTU	MEH	T3	T4
CAS-number:	60-56-0	872-35-5	598-52-7	616-04-6	6893-02-3	51-48-9
Mol. formula:	$C_4H_6N_2S$	$C_3H_4N_2S$	$C_2H_6N_2S$	$C_4H_6N_2O_2$	$C_{15}H_{12}NO_4I_3$	$C_{15}H_{11}NO_4I_4$
Mol. weight:	114	100	90	114	650.9	776.9
Melting point:	146°C	228-231°C	120°C	156°C	236°C	235°C
Water Solubility [mg/l]	$2.75*10^5$	$2.89*10^5$	$2.41*10^5$	$3.09*10^4$	3.96	1.05
pKa (lit. data)	11.5	n.d.	n.d.	9.1	n.d.	n.d.
Log P (lit. data)	-0.34	n.d.	-0.69	-0.91	2.96	4.12
Henry's law constant [atm-m3/mole]:	$2.03*10^{-6}$	n.d.	$3.47*10^{-7}$	n.d.	$3.41*10^{-18}$	$7.91*10^{-19}$
Physical form:	White to yellow crystalline powder	Off-white to light yellow crystalline	White crystalline powder	White crystalline solid	Colourless to off-white crystalline	Off-white crystalline
Vapor pressure [mm Hg]:	$3.28*10^{-4}$	n.d.	2.22	n.d.	$3.9*10^{-16}$	$1.24*10^{-17}$
Health risk:	Antithyroide agent, carcinogen	n. d.	Teratogene, hepatotoxic, developmental abnormalities	n. d.	n.d.	n.d.

VI. CHROMATOGRAPHIC PROCEDURES FOR THE DETERMINATION OF THE TANAKA DIAGRAM PARAMETERS

1. *Amount of alkyl chains*

10 µl of the test solution, containing 0.6 mg/ml amylbenzene dissolved in 80% MeOH/water, was injected. The corrected capacity factor and the column efficiency at 50% peak height were calculated.

2. *Hydrophobicity*

10 µl of the test solution, containing 0.6 mg/ml amylbenzene and 0.4 mg/ml butylbenzene dissolved in MeOH, were injected. The quotient of the corrected capacity factor of amylbenzene to that of butylbenzene was calculated.

3. *Steric selectivity*

10 µl of the test solution, containing 0.5 mg/ml triphenylene and o-terphenyl in methanol, was injected. The quotient of the corrected capacity factor of triphenylene to that of o-terphenyl was calculated.

4. *Hydrogen bonding capacity*

A mobile phase containing 30% methanol/water was employed. 10 µl of the test solution, containing 0.5 mg/ml caffeine and 1 mg/ml phenol in methanol, was injected. The quotient of the corrected capacity factor of caffeine to that of phenol was calculated.

5. *Ion exchange capacity at pH >7*

A mobile phase containing 0.02 M KH_2PO_4, pH 7.6 in 30% methanol/water was employed. 20 µl of the test solution, containing 0.5 mg/ml benzylamine and 0.5 mg/ml phenol in methanol, was injected. The quotient of the corrected capacity factor of benzylamine to that of phenol was calculated.

6. *Ion exchange capacity at pH<3*

A mobile phase containing 0.02 M KH_2PO_4, pH 2.7 in 30% methanol/water was employed. 20 µl of the test solution, containing 0.5 mg/ml benzylamine and 0.5 mg/ml phenol in methanol, was injected. The quotient of the corrected capacity factor of benzylamine to that of phenol was calculated.

All characterisation tests had the following parameters:

Mobile phase was 80% methanol/water with 1.5 ml/min flow rate in a 20 min total run time.

Detection was carried out at 254 nm, column temperature was 40°C.

VII. FISH SAMPLE CODES EXPOSED TO METHIMAZOLE

Sample remark	Exposition concentration [µM MET]	Duration of the exposition [days]	Sex
1-3	Control	15 days	W
4-8	control	15 days	M
9-13	10	15 days	W
14-19	10	15 days	M
20-23	50	15 days	W
24-26	50	15 days	M
27-31	100	15 days	W
32-35	100	15 days	M
36-39	300	15 days	W
40-43	300	15 days	M
44-46	900	15 days	M
B1-5	Control	10 days	W
B6-10	10	10 days	W
B11-15	50	10 days	W
B16-20	100	10 days	W
B21-25	300	10 days	W
B26-30	Control	10 days	M
B31-35	10	10 days	M
B36-40	50	10 days	M
B41-45	100	10 days	M
B46-50	300	10 days	M
B51-55	900	10 days	M
A1-A5w	Control	5 days	W
A1-A5m	Control	5 days	M
B1-B5w5	10	5 days	W
B1-B5m5	10	5 days	M
C1-C5w	50	5 days	W
C1-C5m	50	5 days	M
D1-D5w	100	5 days	W
D1-D5m	100	5 days	M
E1-E5w	300	5 days	W
E1-E5m	300	5 days	M
F1-F5m	900	5 days	M

VIII. SYNTHESIS OF MSOH AND MSO2H STANDARDS

10 mg methimazole was dissolved in 10 ml 0.1 M NaOH, pH 13. 120 µl 30% hydrogen-peroxide was then added to the solution. The mixture was left at room temperature for 48 hours. Reaction progress was monitored by HPLC method for methimazole analysis. After 48 hours methimazole peak was totally disappeared and the reaction product concentration and ratio was determined by calculating of the product peak areas based on the known methimazole peak area as initial reaction parameter. Average product ratio was 45% MSOH and 55% MSO2H. After verifying reaction products, the mixture was neutralised by 1M HCl. This solution was used as stock solution for the measurements.