

Development of Analytical Methods to Quantify Thyroid Hormones (TH) in Multiple Biological Matrices to Facilitate the Prediction of Endocrine Disruption in the Testing and Assessment of Chemicals

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Eidesstattliche Erklärung

Hiermit erkläre ich, Christiane Hindrichs, dass ich die vorliegende Dissertation mit dem Titel
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Biological Matrices to Facilitate the Prediction of Endocrine Disruption in the Testing and
Assessment of Chemicals“

eigenständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Kooperationen mit anderen Mitarbeitern von anderen Abteilungen wurde benannt. Einzelne Teile der Dissertation wurden als Publikationen, Vorträge und Postern bereits veröffentlicht, welche auf den nächsten Seiten aufgelistet wurden. Die Dissertation liegt bei keinem anderen Fachbereich oder einer anderen Hochschule als Prüfungsarbeit vor, bzw. es wurde kein anderes Promotionsverfahren beantragt.

Kaiserslautern, den

Christiane Hindrichs

List of Publications

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Summary

The thyroid hormone (TH) synthesis is regulated by negative feedback mechanism involving the hypothalamus, pituitary gland and thyroid gland which is called the hypothalamic-pituitary-thyroid (HPT)-axis. This HPT-axis flexible adjusts to the T4 and T3 concentrations in blood to either stimulate or repress TH synthesis. THs are important regulators for various processes such as metabolic rate and thermogenesis but also play an important role during development in particular of the neuronal system. Within this TH homeostasis TH-related disruption might occur through various mode of actions (MOAs) by e.g. industrial chemicals, drugs, or pesticides. The effect on TH concentrations in rat plasma as well as brain by different TH-related MOAs caused by representative substances were investigated during the scope of this project. To assess these alterations, first method development was conducted to detect THs (T4, T3, rT3, T2, and T1) in the given matrices using an online-SPE-LC-MS/MS system. The investigated MOAs were divided by either directly interfering with the TH system through e.g. inhibition of the thyroid peroxidase (TPO) directly affecting TH synthesis or indirectly such as by competitively binding to plasma TH-binding proteins. The shown results indicated the strongest decline of TH concentrations after exposure to substances directly interfering with the TH synthesis which was evident in most of the analyses investigated. Additionally, the free fraction of TH in blood was assessed for which first different technical devices were tested from which one was further methodically improved. As a final step the free fraction in plasma samples from rats exposed to different TH disrupting substances were determined. TH analysis in rat plasma was further expanded to TH detection in rat whole brain as well as in specific brain regions. In rat plasma and rat brain the course of TH concentration with increasing age (post-natal day (PND) 4, 21, and 97-99 day-old) was investigated and contrasted between the matrices. This revealed for example peak concentrations of analyte T3 in both matrices and of T2 in brain on PND21. Moreover, *in-vitro* analysis of rT3 to T2 conversion was assessed in human liver microsomes via online-SPE-LC-MS/MS analysis. The potential interference by exemplary substances with the responsible deiodinase enzyme (DIO1) was evaluated by calculating the concentration at which half of the enzyme is inhibited (IC_{50}). These results were compared to the initial method evaluating the release of Iodide by a colorimetric reaction. The assessment of rT3 and T2 concentrations by online-SPE-LC-MS/MS analysis was advantageous especially when assessing the influence of substances structurally containing Iodide. Furthermore, it has been identified that T2 concentrations were a superior readout

parameter than rT3 because it demonstrated to be independent of handling errors that were visible in rT3 concentrations.

Besides TH analysis in rats, the developed method was further adjusted to assess TH whole embryo content in zebrafish (*Danio rerio*). Zebrafish embryos are alternative models because below 120 hours post fertilization (hpf), they do not account to animal welfare regulations and current research showed that until this age they seem to be a suitable model to assess thyroid disruption. Thus, after primarily analyzing THs in control zebrafish samples to determine the number of embryos needed as well as secondly to assess the course of TH concentrations over time. An exemplary study was performed exposing the embryos to the positive substance 6-Propy-2-thiouracil (PTU) which is a thyreostatic drug directly inhibiting TPO demonstrated reduced T4 and T3 concentrations even after beginning of TH synthesis (~ 70 to 80 hpf).

All in all, an easily adjustable online-SPE-LC-MS/MS method was developed which was easily modified for TH analysis of various tissues *in-vivo* and *in-vitro*. This expansion to various matrices of different origin enables a TH modality assessment of various MOAs within the TH system.

Zusammenfassung

Die Thyreoid Hormon (TH, auch Schilddrüsenhormon) Synthese wird über die Hypothalamus-Hypophysen-Thyreoid-Achse (HPT-Achse) anhand der T4 and T3 Konzentrationen im Blut reguliert. An verschiedenen Stellen im TH System sowie über verschiedene Wirkmechanismen können z.B. Medikamente, Pestizide oder Industriechemikalien eingreifen und das Gleichgewicht des Systems stören. Diese Mechanismen interferieren entweder direkt über z.B. die Inhibition der Thyreoidperoxidase (TPO) oder indirekt durch das kompetitive Binden an die Thyreoid Transportproteine im Blut. Verschiedene repräsentative Substanzen verschiedener Wirkmechanismen wurden während dieser Arbeit untersucht bzgl. ihres Effektes auf die TH Konzentrationen im Plasma von exponierten Ratten. Die Ergebnisse zeigten den stärksten Effekt im Plasma nach Exposition von TPO Inhibitoren im Vergleich zu Substanzen mit indirekten Effekt. Zusätzlich zur Analyse der Gesamt-TH Konzentration wurde auch die Konzentration des freien TH-Anteils bestimmt. Hierzu wurden verschiedene Techniken zur Bestimmung des freien Anteils zunächst getestet und anschließend eine Methode weiterentwickelt. Im finalen Schritt wurden Plasmaproben von mit einem TPO-Inhibitor exponierten Ratten gemessen. Die TH-Analyse wurde weiter expandiert für die Matrix Rattenhirn sowie für spezifische Hirnregionen. Hierfür wurden Hirnproben von Ratten verschiedenen Alters bzgl. ihrer TH-Konzentrationen gemessen und dem Ergebnis der jeweiligen Plasmaprobe gegenübergestellt. Dies zeigte eine altersabhängige Schwankung der TH-Konzentrationen in Rattenhirn sowie Rattenplasma, welches sich auch in der Analyse einzelner Hirnregionen wiedergespiegelt hat. Wie zum Beispiel, dass T3 die höchste Konzentration in beiden Matrices und T2 im Gehirn an postnatal Tag 21 aufwies. Ein weiterer Ansatz war die *in-vitro* Bestimmung der rT3 zu T2 Konversion in humanen Lebermikrosomen mittels online-SPE-LC-MS/MS Analyse. Verschiedene potentielle Inhibitoren wurden getestet und wenn möglich die mittlere inhibitorische Konzentration (IC₅₀) bestimmt. Anhand diesem ermittelten Wertes wurden die Ergebnisse mit den Werten der kolorimetrischen Bestimmung verglichen. Die entwickelte online-SPE-LC-MS/MS Methode wies einige Vorteile auf, wenn zum Beispiel Iod-haltige Substanzen untersucht werden müssen. Des Weiteren erwies sich T2 als stabiler Auswerteparameter, da er nicht direkt von Pipettierfehlern wie z.B. rT3 abhängig ist. Im Verlauf des Projektes wurden weitere Organismen untersucht wie zum Beispiel Zebrafisch (*Danio rerio*) Embryonen. Kinetische Analysen der TH Konzentrationen wurden alle 24 Stunden nach Fertilisierung (hpf) bis 120 hpf durchgeführt. Da bis zu diesem Zeitpunkt kein Tierversuchsantrag benötigt wird, gelten die Zebrafish Embryonen als Alternativmodel. Bisherige Forschung mit Zebrafish Embryonen hat

bereits gezeigt, dass es sensitiv für die Bewertung von Thyreoid Disruption ist. Im zweiten Schritt wurden die Embryonen über mehrere Tage mit 6-Propy-2-thiouracil (PTU) exponiert, um zu prüfen in wiefern im frühen Stadium ein thyreoid-relevanter Effekt detektiert werden kann. Die Ergebnisse zeigten, dass auch nach Beginn der TH Synthese in Zebrafisch Embryonen (~ 70-80 hpf), die mit PTU exponierten Embryonen reduzierte T3 und T4 Konzentrationen aufwiesen.

Im Großen und Ganzen wurde eine robuste online-SPE-LC-MS/MS Methode entwickelt zur Analyse von TH Konzentrationen in verschiedenen Matrices. Diese Methode ist flexibel anpassbar für weitere Matrices *in-vivo* sowie *in-vitro* mit minimalem Aufwand, welches im Verlauf des Projektes gezeigt wurde. Dadurch ist es möglich Veränderungen durch verschiedene Wirkmechanismen im TH System zu analysieren.

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List of Abbreviations

3-BPA	3-Phenoxybenzoic-Acid
3-MC	3-Methylcholanthrene
AA	Acetic Acid
AChE	Acetylcholinesterase
ACN	Acetonitrile
AOP	Adverse Outcome Pathway
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BBB	Blood Brain Barrier
BPA	Bisphenol A Diglycidyl Ether
CAD	Collisional Activated Dissociation
cAMP	Cyclic Adenosine Monophosphate
CE	Collision Energy
CLP	Classification, Labelling and Packaging
CNP	2,3-Cyclic Nucleotide 3-Phosphodiesterase
CNS	Central Nervous System
cps	Count Per Second
CSF	Cerebrospinal Fluid
CUR	Curtain Gas
d	day
DG SANTE	Directorate-General for Health and Food Safety
DIO1, DIO2, and DIO3	Iodothyronine Deiodinase Types I, II and III
DIT	Diiodotyrosine
EATS	Estrogenic, Androgenic, Thyroid, and Steroidogenic
EBDC	Ethylene Bisdithiocarbamate
ECHA	European Chemicals Agency
ED	Endocrine Disruption/Disruptor
ED HH	ED for Human Health
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EQD	Equilibrium Dialysis
EtOH	Ethanol
ETU	Ethylene Thiourea
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FA	Formic Acid
FF	Formalin-fixed
ft3	Free Triiodothyronine
ft4	Free Thyroxine/Tetraiodothyronine
fTH	Free Thyroid Hormone
GD	Gestational Day
GS1	Gas 1
GS2	Gas 2
HD	High Dose
HLB	Hydrophilic–Lipophilic Balance
HPD	High Pressure Dispenser
hpf	Hours Post Fertilization
HPT axis	Hypothalamic-Pituitary-Thyroid Axis
ID	Iodine Deficiency
IDD	Iodine Deficiency Disorder
IRD	Inner Ring Deiodination
IS	Internal Standard
IS-mix	Isotope-Labelled Internal Standard Mix
JRC	Joint Research Centre
K_M	Michaelis-Menten Constant

List of Abbreviations

<i>LAT</i>	L-Type Amino Acid Transporters
<i>LC</i>	Liquid Chromatography
<i>LC-Q-TOF MS</i>	LC-Quadrupole Time-Of-Flight MS
<i>LD</i>	Low Dose
<i>LDL-R</i>	Low-Density Lipoprotein Receptor
<i>LLE</i>	Liquid-Liquid-Extraction
<i>LOD</i>	Limit of Detection
<i>LOQ</i>	Limit of Quantification
<i>MAG</i>	Myelin-Associated Glycoprotein
<i>MBP</i>	Myelin Basic Protein
<i>MCT8</i>	Monocarboxylate Transporter 8
<i>MeOH</i>	Methanol
<i>MIT</i>	Monoiodotyrosine
<i>MMI</i>	Methimazole
<i>MOA</i>	Mode of Action
<i>MP-A/-B</i>	Mobile Phase-A/-B
<i>MRM</i>	Multiple Reaction Monitoring
<i>MS</i>	Mass Spectrometry
<i>MWCO</i>	Membrane Molecular Weight Cut-Off
<i>NAM</i>	New Approach Methodologies
<i>ND</i>	No Data
<i>NHANES</i>	National Health and Nutrition Examination Survey
<i>NIS</i>	Sodium-Iodide Symporter
<i>NS diet</i>	Non-Supplemented Diet
<i>OATP</i>	Organic Anion-Transporting Polypeptides
<i>OC</i>	Organochlorines
<i>OECD</i>	Organisation for Economic Co-operation and Development
<i>OP</i>	Organophosphates
<i>ORD</i>	Outer Ring Deiodination
<i>PAPS</i>	3'-Phosphoadenosine-5'-Phosphosulfate
<i>PBDE</i>	Polybrominated Diphenyl Ether
<i>PBS</i>	Phosphate Buffered Saline
<i>PCB</i>	Polychlorinated Biphenyl
<i>PCN</i>	Pregnenolone-16 α -carbonitrile
<i>PE</i>	Paraffin-embedded
<i>PFHxS</i>	Perfluorohexanesulphonic Acid
<i>PKA</i>	Protein Kinase A
<i>PLP</i>	Proteolipid Protein
<i>PND</i>	Post-Natal Day
<i>PTU</i>	6-Propyl-2-thiouracil
<i>PVH</i>	Paraventricular Nucleus of the Hypothalamus
<i>RED</i>	Rapid Equilibrium Dialysis
<i>RIA</i>	Radioimmunoassay
<i>RSD</i>	Relative Standard Deviation
<i>RT</i>	Room Temperature
<i>rT3</i>	Reverse Triiodothyronine
<i>RXR</i>	Retinoid X Receptor
<i>S Diet</i>	Supplemented Diet
<i>SBH</i>	Subcortical Band Heterotopia
<i>SD</i>	Standard Deviation
<i>SK</i>	Sandell-Kolthoff
<i>SPE</i>	Solid Phase Extraction
<i>SREBP-2</i>	Sterol Response Element Binding Protein-2
<i>SULT</i>	Sulfotransferase
<i>T1</i>	Monoiodothyronine
<i>T2</i>	Diiodothyronine
<i>T3</i>	Triiodothyronine
<i>T4</i>	Thyroxine/Tetraiodothyronine
<i>TBBPA</i>	3,3',5,5'-Tetrabromobisphenol A

List of Abbreviations

<i>TBG</i>	Thyroxine-Binding-Globulin
<i>TETRAC</i>	3,3',5,5'-Tetraiodothyroacetic acid
<i>Tg</i>	Thyroglobulin
<i>TH</i>	Thyroid Hormones
<i>THD</i>	Thyroid Hormone Disruption/Disruptor
<i>TMTU</i>	Tetramethyl Thiourea
<i>TPO</i>	Thyroid Peroxidase
<i>TR</i>	Thyroid Hormone Receptor
<i>TRE</i>	Thyroid Response Element
<i>TRH</i>	Thyrotropin-Releasing Hormone
<i>TSH</i>	Thyrotropin-Stimulating Hormone
<i>TSHr</i>	Thyrotropin-Stimulating Hormone Receptor
<i>TTR</i>	Transthyretin
<i>UDPGA</i>	UDP-Glucuronic Acid
<i>UF</i>	Ultrafiltration
<i>UGTs</i>	UDP-Glucuronyltransferases
<i>USI</i>	Universal Salt Iodization
<i>VC</i>	Vehicle Control
<i>WHO</i>	World Health Organization

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1 Introduction

The World Health Organization (WHO) defines Iodine deficiency disorders (IDD) as “all of the consequences of Iodine deficiency (ID) in a population that can be prevented by ensuring that the population has an adequate intake of Iodide”¹. IDD has a spectrum of different health consequences such as goiter, hypothyroidism, delayed physical development, mental deficiency, and impaired mental function additionally depending on the age, when ID occurs. Therefore recommended daily Iodide intake concentrations were established which suggest 90 µg for preschool children (0-59 m), 120 µg for schoolchildren (6-12 y), 150 µg for adults (> 12 y) , and 250 µg for pregnant and lactating women¹. Higher Iodide intake is crucial for pregnant woman because the fetus is fully dependent on maternal thyroid hormone (TH) supply since it cannot synthesize them on its own. Sufficient concentration of THs ensure optimal physical development especially of the fetal brain and thus should be ensured. As a prophylaxis to prevent IDD universal salt Iodization (USI) was internationally recommended and has been embedded in many national programs¹. Iodide intake is monitored by measuring urinary Iodide concentration which should range from 100-199 µg/L in school-aged children¹. Effectiveness of USI is reflected in the number of countries with adequate Iodide intake which has increased from 67 to 118 countries in the period from 2003 to 2020². However, insufficient Iodide intake is still present in around 21 countries independent of their economic status as the reasons range from failure of the salt Iodization program due to politically instability, natural disaster and war, and lack of usage of Iodized salt in the production of processed foods^{2,3}. Another factor is the growing interest of reducing salt intake to prevent hypertension which goes along with a decrease Iodide intake therefore Iodide concentration in salt should be increased to ensure recommended daily intake concentration². ID ultimately leads to hypothyroidism which is also induced by other causes such as for example chronic autoimmunity diseases (e.g. Hashimoto thyroiditis), pituitary or hypothalamic dysfunction, genetic mutations (e.g. TH transporter or metabolism genes) or induced by xenobiotics⁴. These diseases might lead to hypothyroidism through different mode of actions (MOAs) within the TH system such as directly by inhibiting TH synthesis or indirectly through binding to enzymes responsible for TH conversion or to the plasma TH-binding proteins which will be subsequently discussed further.

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

The production of THs (tetraiodothyronine (T4) which is also referred to as thyroxine and triiodothyronine (T3)) is regulated by the hypothalamic-pituitary-thyroid (HPT) feedback mechanism. The key regulator hereby is the bioactive T3 which binds to the thyrotropin-releasing hormone (TRH) located in the paraventricular nucleus of the hypothalamus (PVH) when TH concentrations are low⁵. TRH is a tripeptide (pyroGlu-His-ProNH₂) cleaved from its prohormone in the hypothalamus. The preprohormone (preproTRH) encodes for around 29 kDa including five copies of the prohormone TRH sequence in the rat and mouse whereas in human it is six copies⁶⁻⁸. The preproTRH is further cleaved and modified via cyclization at the N-terminus and amidation at the C-terminus by two convertases proprotein convertase-1 (PC-1) and PC-2 and carboxypeptidase E to yield mature TRH⁹⁻¹¹. The mature TRH migrates from the paraventricular nucleus of the TRH neurons further to the pituitary gland where it binds to the cell-surface TRH-R1 receptors on thyrotrophic cells to induce production of thyrotropin or also namely thyroid-stimulating hormone (TSH)¹². TSH secretion is also controlled by T4 and in particular T3. Within this feedback mechanism T3 is in charge of the expression of genes for TRH-R1 as well as for the transcriptional inhibition of TSH subunits¹³. The TSH subunits are divided into α - and β - subunits, whereas the α -subunit is identical to other hormones such as luteinizing and follicle stimulating hormone¹⁴. Upon induction of TSH production through TRH, TSH is primarily released into the circulation and binds to its TSH receptor (TSHr) in the thyroid gland. The TSHr is a member of the seven-transmembrane spanning G protein-coupled receptor family and its activation responds to activate the adenylyl cyclase, increase intracellular concentration of cyclic adenosine monophosphate (cAMP) which is followed by activation of protein kinase A (PKA)^{14, 15}. The biosynthesis of THs further involves the uptake of Iodide from plasma into the thyroid follicular cells via the Sodium Iodide symporter (NIS). The K_M value refers to the affinity of an enzyme towards a given substrate and the value depicts the concentration of the substrate after half of the reaction rate is completed which is for the transport of Iodide 10-30 μ M compared to 46-60 nM for Sodium ions and acquires an approximately 30-60-fold higher Iodide concentration in the thyroid than in blood¹⁶⁻¹⁸. The Iodide is further transported into the colloid across the apical membrane via the pendrin transporter where it is oxidized to Iodine by the thyroid peroxidase (TPO). As a next step the tyrosyl residues of thyroglobulin (Tg) are iodinated in the presence of hydrogen peroxide (H₂O₂) by TPO forming monoiodotyrosine (MIT) or diiodotyrosine (DIT) residues on Tg depending on the number of Iodide atoms attached. MIT and DIT function as precursor hormones of T4 and T3 which are formed after phenolic coupling of these Iodotyrosyl residues

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

by TPO. T4 is formed by coupling of two diiodotyrosyl residues and T3 by one monoiodotyrosyl and one diiodotyrosyl residue. The Tg bound with T4 and T3 is transferred back into the follicular cells through endocytosis where it undergoes proteolysis in the lysosomes to release T4 and T3. Uncoupled monoiodo-, and diiodotyrosyl residues will be removed from Tg and deiodinated by flavoprotein iodotyrosine deiodinase to save Iodide for the next TH biosynthesis (TH synthesis is illustrated in Figure 1 below)¹⁸⁻²⁰.

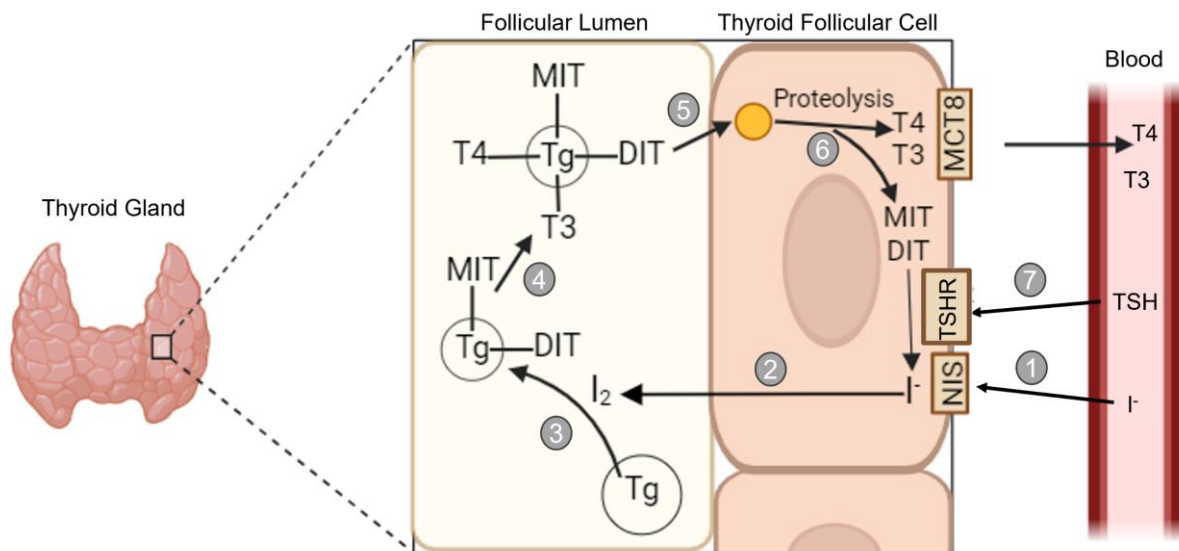


Figure 1. Schematic illustration of TH synthesis in the thyroid gland.

1) Uptake of Iodide via NIS into the thyroid follicular cell. 2) Iodide enters the follicular lumen via the pendrin transporter after which it is oxidized by TPO in the presence of H_2O_2 . 3) Tg is stored in the follicular lumen. In the presence of TPO and H_2O_2 its residues are Iodinated forming MIT and DIT. 4) Phenolic coupling of these Iodotyrosyl residues by TPO further form T4 or T3. 5) The bound Tg (depicted as a yellow circle) is transported into the follicular cell by endocytosis 6) in which T4 and T3 are liberated from Tg by undergoing proteolysis. Remaining MIT and DIT residues are also removed from TG and further deiodinated. T4 and T3 enter the blood stream through MCT8. 7) TSH binding to its TSH receptor stimulating TH synthesis. Created in <https://BioRender.com>.

The liberated T4 and T3 enter the circulation through the monocarboxylate transporter 8 (MCT8) from where they are distributed into different tissues which they enter via the same transporter and/or via the organic anion-transporting polypeptides (OATP) transporters. The OATPs are a gene superfamily of transport proteins expressed in different tissues as for example OATP1C1, which is orthologous expressed in rat and human and is inter alia responsible for the TH uptake into the brain preferentially of T4^{21,22}. Human placental transfer of THs involve some of the following transporters: MCT-8, OATP4A1 and L-type amino acid transporters (LAT1 & 2)^{22, 23}. In the blood THs are mainly bound to different plasma transport proteins (> 99 %) such as thyroxine-binding-globulin (TBG), transthyretin (TTR) and albumin whereas the relative percentage of carrying T4 depends on e.g. binding affinity, concentration of the

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

proteins in blood and free T4 concentration²⁴. This leads for example in human to approximately 75 % of total T4 bound to TBG, 20 % to TTR and 5 % to albumin²⁵. Similar to human, the highest binding affinity sites towards T4 and T3 is found for TBG in the rat, present only in rat offspring with expression peaks at around postnatal days 21-23. From these days onwards TBG concentration decreases reaching the lowest and insignificant concentration in the adult rat (~ 60 days old)^{26, 27}. The small unbound fraction so called “free” THs (fTH) are approximately 0.03 % of total T4 and 0.3 % of total T3 in serum and this fraction is able to enter different target cells where it binds to its thyroid hormone receptor (TR) to mediate gene transcription¹⁴. There are multiple TR isoforms, but all have a similar domain structure consisting of an amino-terminal A/B domain, a central DNA-binding domain (DBD) containing two “zinc fingers”, a hinge region containing the nuclear localization signal and a carboxy-terminal ligand binding domain (LBD)¹⁴. The two major isoforms are TR α and TR β which are encoded on separate genes and its TH-regulated gene expression is cell type dependent²⁸. These isoforms are separated into additional TRs by alternative splicing whereas the main isoforms are TR α -1, TR β -1 and TR β -2²⁹. TR α results into TR α -1 and *c-erbA α -2* protein, the latter binds weakly to the thyroid response element (TRE), but cannot lead to transcription of TH-responsive genes and thus it will not be further explained in detail¹⁴. The TR β isoform contains two promoter region which are important for distinct mRNA transcription for specific proteins. Depending on which promoter region is activated, the TR β isoforms TR β -1 and TR β -2 are generated. The two TR β s bind to the TRE and to TH with high affinity and show identical DNA-binding domain, hinge region and LBD, but they distinguish in the amino-terminal regions¹⁴. The different TRs bind to specific TREs in the promoter region of the given T3-target genes leading to either activation or repression of transcription³⁰. This TR-binding to TREs occurs majorly as heterodimer with retinoid X receptor (RXR)³⁰. T3-ligand-bound TR causes conformational change within the receptor which consecutively releases corepressors and coactivators²⁹. Repression of transcription can occur when unliganded TRs bind to TREs or when ligand-bound TRs bind to negative TREs²⁹. All isoforms are expressed in almost all tissues, but the highest expression of TR α -1 can be found in skeletal muscle and brown adipose tissue (BAT), TR β -1 in brain, liver, and kidney and solely TR β -2 is tissue specific expressed in anterior pituitary gland, areas of the hypothalamus, in developing brain and inner ear^{14, 28, 31, 32}.

1.1.1 Metabolism of Thyroid Hormones in Tissue and Species Differences

Since T3 mainly binds to TR, local conversion from T4 to T3 in tissue is needed to conduct TH signaling. The enzymes responsible for TH conversion are the selenoenzyme Iodothyronine

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

deiodinase types I, II and III (DIO1, DIO2, and DIO3) which properties are enumerated in Table 1. Dependent on the DIO type the TH conversion is either activating or inactivating dependent on which TH is the product and on the deiodination of the outer (ORD) or inner ring (IRD). DIO3 inactivates T4 to reverse triiodothyronine (rT3) or T3 to diiodothyronine (T2). It is expressed in the placenta or the central nervous system (CNS) and plays a role in clearance of T4 and T3 as well as it prevents further T3 production³³. The DIO type II can be also found in the CNS and placenta and additionally in the pituitary gland as well as in BAT. Since it has activating properties converting T4 to the bioactive T3, it is the major source for T3 in plasma and intracellular in tissue. DIO1 on contrary functions as activating and inactivating (T4 to T3, T3 to T2, and rT3 to T2), but its preferred substrate is rT3 and it is expressed among other organs in the liver and kidney. An overview of the different DIO types as well as their corresponding binding affinities are enumerated in Table 1. In addition to excretion via conversion by DIOs, THs can be metabolized by glucuronidation and sulfation of the phenolic hydroxyl group. Sulfotransferases and glucuronidases enzymes count to phase II detoxification reactions as they increase water solubility and molecular weight of a compound to facilitate urinary or biliary clearance³⁴. TH sulfates are easily degraded by DIO1 and thus its plasma, bile and urine concentrations are rather low when DIO1 activity is high^{35, 36}. Sulfotransferase (SULT) enzyme family divides itself into different subfamilies as for example the phenol sulfotransferases (SULT1 family) and hydroxysteroid sulfotransferases (SULT2 family) which are based on their substrate specificity. They transfer a sulfate group from its cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to e.g., a hydroxyl group of the substrate and are located in the cytoplasmic fraction of the liver, kidney, intestine, and brain³⁴. For different enzymes from the human SULT1 family an activity towards TH has been determined as for example SULT1A1, 1A3 and even the estrogen sulfotransferase SULT1E1 catalyzes the sulfation of TH³⁷⁻³⁹. In rats it has been detected that sulfonation stimulates IRD of sulfated T4 by 200-fold compared to non-sulfated T4 deiodination by DIO1, but it seems to have an inhibitory to no effect on ORD of other substrates³⁵. Sulfated TH serum concentrations are high in fetal and cord blood compared to normal human concentrations; it is speculated that fetus' sulfated T3 functions as a reservoir for fast tissue specific T3 release^{35, 40}. On the contrary, glucuronidated THs are rapidly excreted in the bile as well as they can be reabsorbed in the intestine after hydrolysis by bacterial β -glucuronidases³⁴. Ultimately around 20 % of daily T4 production is excreted in the feces³⁴. The enzymes responsible for glucuronidation are UDP-glucuronyltransferases (UGTs) transferring a glucuronic acid group from its cofactor UDP-glucuronic acid (UDPGA) to a hydroxyl or carboxyl group to increase the substrates' water-

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

solubility. They are located in the endoplasmic reticulum of mainly liver, kidney and intestine³⁴. Like SULTs, UGTs are divided into two further subfamilies UGT1A and UGT2B, however glucuronidation seems to be more important in rodents than in humans³⁴. Additional species differences are that adult rats lack the high affinity thyroid-binding protein TBG, have much lower half-lives of T4 and T3 (e.g.: 12-24 h vs. 5-9 d for T4) compared to humans, and the enterohepatic circulation of TH glucuronides is assumed to be less effective^{41, 42}.

Table 1. Overview of the three types of selenoenzyme Iodothyronine deiodinases.

The table was adapted and information combined from Peeters and Visser as well as Bianco and Kim^{33, 34}.

DIO Type	DIO1	DIO2	DIO3
	<pre> graph TD T4 --> T3 T4 --> rT3 T3 --> T2 rT3 --> T2 </pre>	<pre> graph TD T4 --> T3 rT3 --> T2 </pre>	<pre> graph TD T4 --> rT3 T3 --> T2 </pre>
	ORD and IRD	ORD	IRD
Tissues	Liver, Kidney, Thyroid Gland	CNS, Pituitary Gland, BAT, Skeletal Muscle, Placenta	CNS, Placenta, Fetal Tissues
Preferred Substrates	rT3 >> T4 ≈ T3	T4 > rT3	T3 > T4
Km values	≈ 0.1-10 μM	≈ 1 nM	≈ 10 nM
Physiological Role	Clearance of rT3, Plasma T3 production	Development, Thermogenesis, intracellular T3 production	Clearance of T3 and T4, Development
Hypothyroidism	Decrease	Increase	Decrease
Hyperthyroidism	Increase	Decrease	Increase

1.1.2 TH-Dependent Role in Biological Processes and Functions

The TH-stimulated gene transcription followed by protein synthesis regulates important functions in various tissues. To name a few example tissues, it has been shown that THs affect the expression of various bone markers in serum reflecting possible changes in bone formation and resorption¹⁴. Since THs influences the circulation and the heart function itself, the overall cardiac output is encumbered. The consequences are for example a high cardiac output for hyperthyroid patients and for hypothyroid patients a respectively low cardiac output, decreased vascular volume and a decreased stroke volume^{14, 43}. Besides the alterations on the overall cardio-vascular system, THs positively and negatively regulate the transcription of several genes for cardiac protein⁴³. In the liver THs are also involved in regulating the stimulation of enzymes responsible for lipogenesis such as dehydrogenase and glucose-6-phosphate, lipolysis, and oxidative processes¹⁴. Additionally, enzymes expression involved in cholesterol

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

metabolism, synthesis, cholesterol ester hydrolase, and cholesterol acyltransferase is TH-dependant¹⁴. Enhanced cholesterol synthesis through a rise of cholesterol uptake is mediated via TH-mediated gene transcription of LDL-R (low-density lipoprotein receptor) gene which in turn is modulated by the TH-mediated induction of sterol response element binding protein (SREBP)-2-gene expression⁴⁴. SREBP-2 is a transcription factor involved in inter alia cholesterol metabolism or fatty acid synthesis⁴⁴. Lastly THs are responsible for the maintenance of various other processes such as adaptive thermogenesis, body weight and of a basal metabolic rate⁴⁴.

1.1.2.1 THs Role in Neurodevelopment

Neuronal development during fetal stages is inter alia fully dependent on the supply of maternal THs. Calvo et al. showed that human fetal tissues in the first trimester are exposed to the same concentration range of free T4 as they are at an adult age and that this exposure of free T4 is depending on the concentration of circulating free T4 of the mother⁴⁵. Until the onset of fetal TH synthesis (at around embryonic day 17-18 in rats & around day 90 in human gestation⁴⁶), the overall total T4 concentration is retained at lower concentration of what is found in adult (both in humans & rodents) to protect the fetus from toxic concentration of maternal TH supply. As mentioned in section 1.1 free THs are less than 1 % of total TH concentration in blood which are able to enter different tissue cells through thyroid hormone transporters as for example the blood brain barrier (BBB) or choroid plexus via MCT8 or OATP1C1 which are the most important transporters in fetal tissue^{21, 47}. When free T4 enters the cerebrospinal fluid (CSF), it binds to TTR which is the dominant carrier in this compartment to provide TH supply and prevent removal from CSF⁴⁸. The role of THs in rat brain development has been further underlined in several studies investigating the effect of developmental TH insufficiency in the rat. The observation of heterotopia was identified as a possible indicator for neurodevelopmental toxicity in rat developmental neurotoxicity (DNT) studies. This has been mainly demonstrated to occur after maternal exposure to 6-Propyl-2-thiouracil (PTU) during gestation causing decreased TH concentrations in offspring, which is required to manifest heterotopia in the offspring (starting exposure from gestation day (GD) 2, but heterotopia was not manifested when the exposure began on PND2)⁴⁹. The authors further reported that the malformations were persistent until PND300 and that no sex difference was detected⁴⁹. In consecutive studies they showed that a PTU exposure period from GD19 to PND2 is sufficient to induce heterotopia more accurately defined as periventricular heterotopia due to hypothyroidism⁵⁰. Periventricular heterotopia is marked as a clustering of neurons which failed to migrate during early development within the periventricular zone located near the lateral

1.1 Synthesis and Regulation of Thyroid Hormones T4 and T3

ventricles. Furthermore, affections of multiple components of cell junctions and of blood-brain as well as blood-cerebrospinal fluid barriers were indicated by e.g. changes in expression patterns of extracellular matrix, and cytoskeletal proteins as well as alterations in filamentous actin, platelet endothelial cell adhesion molecule 1 and claudin 5^{50, 51}. In rats, hypothyroidism causes diminished axonal growth and dendritic arborization in the cerebral cortex, visual and auditory cortex, hippocampus, and cerebellum¹⁴. In cerebellum it additionally delays proliferation and migration of granule cells from the external to the internal granular layer. However, neuronal effects caused by hypothyroidism can be reverted in rats by TH administration within 2 weeks after birth¹⁴. Other clinical outcomes such as subclinical hypothyroidism is defined as elevated TSH concentration with normal T4 and T3 serum concentrations, whereas primary hypothyroidism manifests in low TH serum concentrations because of destruction of the thyroid gland.

The placenta releases only a limited amount of free THs to the fetus via the expression of DIO3 which has been detected in rodent and human placenta, uterus and in fetal tissue^{52, 53}. To highlight the importance of the enzyme DIO3, investigations were conducted using DIO3 knockout mice by Hernandez et al. resulting in neonatal thyrotoxicosis followed by central hypothyroidism which was lifelong persistent and also resulted in abnormalities in the function of the HPT-axis involved tissues^{54, 55}. DIO2 is fundamental in certain periods of development such as its expression strongly increases after birth with its peak around PND28 and then declines until day 50 in rats⁴⁶. An exemplary critical period in which DIO2 is necessary is the maturation of cochlea and thus the onset of auditory function for which T3 must be present. For a better understanding of DIO2 involvement in the developing brain, a DIO2 knockout mice model was examined. Using this model Ng et al. reported that DIO2 is essentially to locally generate T3 in the auditory system because DIO2 knockout mice reached sufficient TH serum concentrations but had impaired auditory function and abnormal cochlear morphology⁵⁶. In another research study mice brain regions (cerebellum, cerebral cortex, hypothalamus, and midbrain) were analyzed for their T3 concentration which was significantly reduced in all regions in the DIO2 knockout mice similar to the wildtype-hypothyroid mice but DIO2 knockout mice yield normal T3 concentration in serum⁵⁷. As indicated with the previously mentioned studies, T3 needs to be locally converted from T4 to initiate target gene transcription. This statement is underlined by research data indicating that up to 80 % of nuclear T3 was locally produced in the cerebellum and cerebral cortex in rats⁵⁸. This statement that T3 concentration is dependent on local conversion in fetal brain was further underlined by a rat study administering Methimazole (MMI) to dams as well as alongside infusion with either T4

1.2 Disruption of TH Homeostasis and Their Consequences

or T3. Examined fetal brain from dams receiving T3 infusion did not increase fetal T3 above the concentration of only MMI-treated rats whereas fetal T3 brain concentrations from dams infused with T4 did reach control concentration when infusing around $\geq 2 \mu\text{g}/100 \text{ g BW}/\text{d}$ ⁵⁹. Additionally, DIO1 to 3 rat expressions change in various fetal tissues during development as for example DIO3 is mostly expressed in fetal liver tissue whereas in several brain tissue all three are detected throughout⁶⁰. On cellular level DIO2 expression was localized almost solely in astrocytes meaning that the predominantly transported T4 from serum is locally converted to T3 which is secondly transferred into neuronal cells via the MCT8 transporter in which it binds to its nuclear receptor TR initiating neuronal gene transcription⁶¹. This initiation in neuronal tissue involves for example Purkinje cell and Bergmann glia differentiation/maturation through activation of TR α -1 in cerebellar development^{62, 63}, astrocyte and neuron differentiation in the hippocampus, oligodendrocyte differentiation and myelination, and synaptogenesis as reviewed in Stepien et al.⁶⁴. One initiation in neuronal tissue is for example the acceleration of myelination by THs. A reduction of myelin mRNA and proteins namely myelin basic protein (MBP), proteolipid proteins (PLP), myelin-associated glycoprotein (MAG), 2,3-cyclic nucleotide 3-phosphodiesterase (CNP) has been detected to delay myelin formation in rat brains of hypothyroid pups^{65, 66}. Furthermore, the number of oligodendrocytes in the white matter tracts corpus callosum and anterior commissure were reduced in hypothyroid offspring⁶⁷. This effect was observed along with reductions in MBP gene expression referring to the previous mentioned alterations to myelination emphasizing TH control during brain development⁶⁷.

1.2 Disruption of TH Homeostasis and Their Consequences

Perturbations of TH concentrations can occur through various modes of action (MOA) at different points in the TH system as pointed out in Figure 2. For example, exposure to thyreostatic drugs, chemicals or Iodide-deficient diet develop different clinical patterns of altered TH concentrations. Maternal Iodine deficiency, if not treated in the right time could lead for example to permanent failure in postnatal neurodevelopment, but also to unwanted adverse effects in adults. Over the years various literature reviews have been conducted evaluating thyroid-disrupting effects of certain chemical classes⁶⁸⁻⁷⁰.

With regards to this project, one crucial point is the interaction with the TH system by these substances in different matrices at various ages, since THs are important for several physiological processes of e.g. metabolism and development especially of the brain^{14, 47, 71, 72}. Chemical substances can cause impairment of the TH system, exemplary substances interfering through different MOAs were analyzed during this work. These chemical substances included for example different thyreostatic drugs, Polychlorinated Biphenyls (PCB), thiocyanates or

1.2 Disruption of TH Homeostasis and Their Consequences

substances from the insecticides classes namely Pyrethroids, Organochlorines (OC), Organophosphates (OP), Phenyl Pyrazoles or Organotin compounds. Some pesticides (mainly the older ones) are ubiquitously found in animals, humans, and in the environment, which is reflected by their half-lives in soil ranging from days to years and posing a potential threat to these organism⁷³, however the assessment of thyroid hormone disruption (THD) is generally of very high priority in Europe (see Chapter 1.3 below). Mode of action investigations in animals suggested various possible mechanisms for thyroid disruption assuming that e.g. OCs due to their structural similarity mimic TH action by binding to thyroid receptor and/or reducing binding to its proteins in plasma⁷⁴. Pyrethroids as well as their prevalent metabolite 3-phenoxybenzoic-acid (3-BPA) have been shown to potential disrupt TH signaling *in-vitro*⁷⁵. Organohalogens especially PCBs and Polybrominated Diphenyl Ether (PBDEs) are structural similar to THs which might explain their interference on various key points in the TH system⁷⁶. These different mechanisms involve competitive binding to plasma TH-binding proteins such as TTR in the bloodstream, inducing phase II metabolism enzymes and thus TH clearance, or enhancing or blocking the production of DIOs as well as the TR⁷⁶⁻⁸¹.

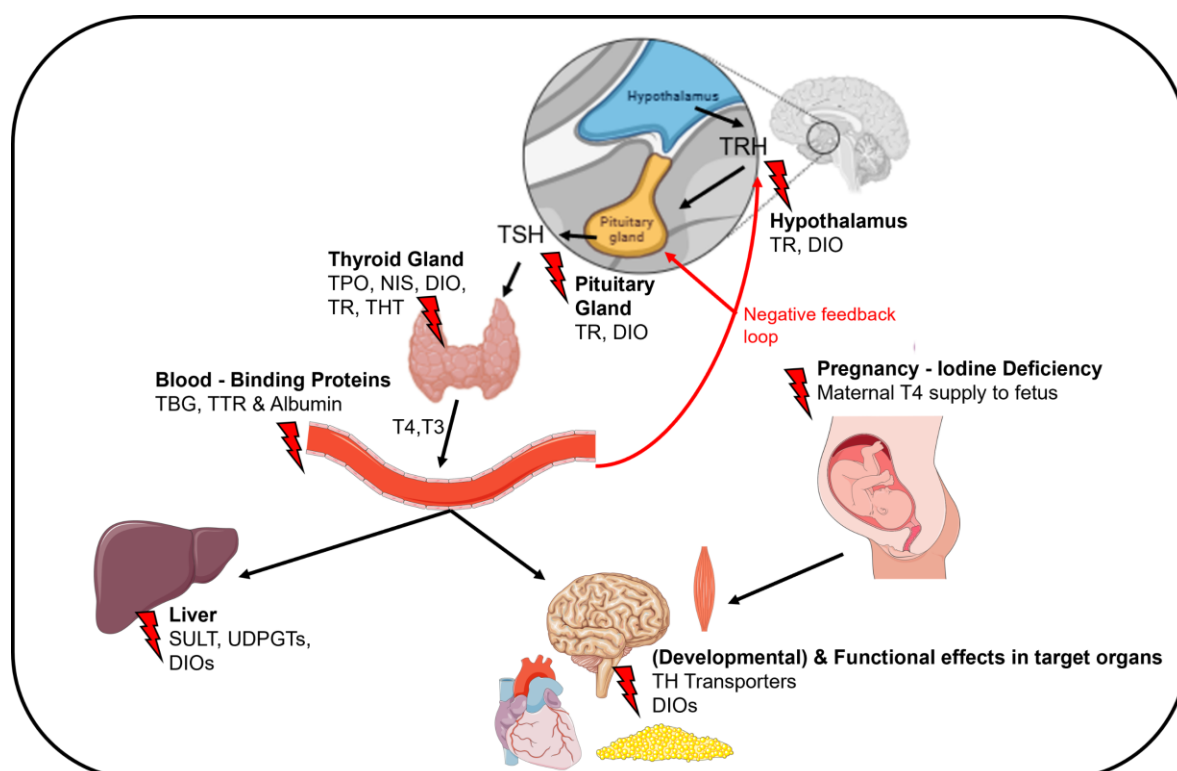


Figure 2. Schematic insight into connectivity of TH system and indication of possible THD interferences. TH signaling of the HPT-axis to target organs are shown by the black arrows. Red lightning symbols refer to possible targets of THD causing functional effects in target organs or increased excretion of TH in the liver. Low Iodide supply during pregnancy causes low maternal TH supply to the fetus which could lead to several developmental impairments. The grey square in the top left corner further illustrates deiodination of THs and the responsible DIO types for each conversion. Adapted and modified from Köhrle and Frädrieh⁸² using images from <https://BioRender.com> and Servier Medical Art licensed under Deed - Attribution 4.0 International - Creative Commons.

1.2 Disruption of TH Homeostasis and Their Consequences

1.2.1 TPO Inhibition Causes Impairment of TH Synthesis

Thyroid Hormone disruptors (THD) such as PTU, Ethylene Thiourea (ETU), and MMI are known to inhibit TPO, thereby causing to terminate the Iodination of tyrosine residues on Tg and their subsequent coupling which finally impairs TH synthesis^{83, 84}. MMI and PTU are thyreostatic drugs administered to treat hyperthyroidism whereas ETU is a metabolite of ethylene bisdithiocarbamate (EBDC) fungicides. In particular PTU is commonly used in basic research to investigate TH regulation on tissue development mainly focusing on the brain due to its direct mechanism on TH synthesis as well as it inhibits DIO1 activity in the liver. To give an example Axelstad et al. studied the effect of PTU on development and possible long-lasting alterations on behavior and hearing in the offspring of Wistar rats. The dams were exposed to PTU from GD7 to PND17 with the aim of causing a temporary hypothyroid state in the fetus which was verified by the reduced T4 concentration in serum with increasing PTU dosage visible in dams on GD15 and in the offspring on PND16 but on PND27 this decline vanished⁸⁵. Long-lasting causes of PTU exposure were visible in the adult offspring such as increased thyroid gland weight, hearing deficits, and hyperactivity correlated with low T4 concentration during development⁸⁵. A PTU-induced maternal hypothyroxinemia rat model highlighted fetal vulnerability towards TH insufficiency indicated by the formation of subcortical band heterotopia (SBH) which persisted until adulthood of the offspring despite returning to euthyroid status⁸⁶. The formation of heterotopia caused by developmental TH insufficiency was further examined in a similar rat model inter alia revealing that exposure starting in gestation led to heterotopia formation but not when exposure began postnatally^{49-51, 87}. Similar to PTU, exposure of MMI in Sprague-Dawley rats during gestation period GD7 to PND22 caused decreased T4 concentration in serum on GD15 and on PND22 in dams and on PND16 in pups with parallel increased TSH concentration resulting in adverse brain development⁸⁸. As mentioned in the second sentence ETU is a metabolite of EBDC such as Mancozeb, exposure to Mancozeb from GD7 to PND16 at doses of either 0, 50, 100, or 150 mg/kg bw/d led to significant dose-dependent decrease of T4 concentrations in dams at GD15 which was not detected in the offspring⁸⁹. Furthermore, the reduced maternal T4 concentrations did not induce behavioral effects in the offspring, but dams in the highest dosage group showed neurotoxic effects such as hind limb paralysis⁸⁹. Lastly, ETU exposure in rats at different ages provoked decrease in T4 and increase in TSH concentration after 9 and 24 months of administration⁹⁰.

1.2.2 Inhibition of Sodium-Iodide Symporter (NIS)

One MOA of disturbance in TH system is the blockage of plasma membrane transport protein Sodium-Iodide symporter (NIS). NIS is responsible for pumping Iodide into the thyroid

1.2 Disruption of TH Homeostasis and Their Consequences

follicular cell in exchange for Sodium ions which guarantees sufficient Iodide concentration for TH synthesis in follicular cells⁷⁶. Furthermore, NIS facilitates Iodide uptake in mammary cells, cervical cells, gastric mucosa, choroid plexus, and saliva¹⁶. Substances that interfere with NIS are e.g.: perchlorate, thiocyanate, and nitrate by blocking the binding site of the transporter so that Iodide cannot be transported into the follicular cell any longer causing decreased TH synthesis⁹¹. Perchlorates have been utilized as oxidizer for example for solid fuel engines, automobile air bags, and fireworks, thus it is a common contaminant in water and soils but can also be found naturally e.g. in Chile^{91, 92}. Especially in the western United States perchlorate contamination was detected in groundwater and rivers from assumably industrial and waste storage sites disposal, in cow milk and human breast milk, and contamination was found in grain, fruit, vegetables, and dietary supplements⁹²⁻⁹⁵. Additional NIS inhibitors are for example nitrate which can be found in lettuce and as a preservative in processed meat and fish, as well as thiocyanate resulting from free cyanide metabolism and from cigarette smoke^{76, 91}. In woman with a lower urinary Iodide concentration of 100 µg/L increasing concentration of urinary perchlorate were correlated significantly with lower serum T4 and higher serum TSH concentrations investigated in the National Health and Nutrition Examination Survey (NHANES) study during 2001-2002 in the United States⁹⁶. This study was further extended to examine a potential correlation between perchlorate, low urinary Iodide, and another inhibitor thiocyanate which is found in cigarette smoke. The authors concluded from the results a possible cumulative effect of thiocyanate and perchlorate because for smoking women with low urinary Iodide the association between urinary perchlorate, decreased T4 in serum, and high urinary thiocyanate was stronger than in non-smoking women and in women with lower thiocyanate concentrations⁹⁷.

1.2.3 Iodine Deficiency Disorders (IDD) – Human Clinical Evidence

During in utero and neonatal neurodevelopment THs play an active and important role to ensure a proper outcome. A key regulator is hereby Iodide which is essential for TH synthesis. Iodide is unevenly distributed in the environment such as soils in mountainous areas are typically Iodide-deficient and the oceans are rather Iodide-rich⁹⁸. Sources for Iodide used to be mainly food and beverages where usually food of marine origin yield the highest amount, however their overall content is low. An overall low intake of Iodide causes low TH concentration which might for example lead to thyroid enlargement (goiter), impaired cognitive function or in severe cases to cretinism. After the discovery that these adversities are caused by ID from the 1970s forward and that they can be prevented by Iodide supplementation, USI was globally embedded as prophylaxis⁹⁸. Cretinism is linked to severe ID and is clinically

1.2 Disruption of TH Homeostasis and Their Consequences

categorized into two groups: neurological and hypothyroid cretinism. “Neurological cretinism” features mental retardation along with a neurological defect such as in hearing and speech or impaired motor activity, but can be euthyroid with low urinary Iodide concentration⁹⁹. The second group “hypothyroid cretinism” is clinically characterized by e.g.: dwarfism, skeletal retardation, sexual retardation, thickened skin or incomplete face maturation caused by severe hypothyroidism⁹⁹. A different consequence of ID or by genetic causes is neonatal hypothyroidism that can result in mental retardation and neurological defects in human. Maternal hypothyroxinemia defines causes that are mainly due to insufficient TH supply by the mother caused by e.g. ID. This could result in various clinical outcomes such as congenital hypothyroidism, miscarriage, stillbirths, goiter, endemic cretinism, or impaired mental function.

In a clinical study embedded in the “Generation R Study” from the Netherlands low and high maternal free T4 concentration were each correlated with a statistically decrease in IQ of the child as well as negatively associated with child grey matter volume which was assessed by magnetic resonance imaging (MRI)¹⁰⁰. This study was embedded in three cohort studies involving Generation R (Netherlands), the INfancia y Medio Ambiente Project (INMA; Spain, three regions), and the Avon Longitudinal Study of Parents and Children (ALSPAC; United Kingdom) to assess association between maternal Iodide status in pregnancy with child IQ. Evaluation of all three cohorts further confirmed the association between Iodide status in pregnancy with child IQ scores in particular the development of verbal IQ of the fetus is vulnerable until start of the second trimester¹⁰¹.

1.2.3.1 Animal Studies Concerning Iodine Deficiency

The effect of ID in different extents from mild to severe have been studied in various animal studies. Usually, pregnant rats are given diets with different Iodide concentration and afterwards the thyroid status of the offspring will be assessed. One of the first studies showing the adversity of maternal hypothyroxinemia was conducted by Lavado-Autric et al. who reported alterations of the histogenesis and cytoarchitecture of the rat fetal cerebral cortex⁷¹. Liu et al. exposed Wistar rats to either low-Iodide or marginal Iodide deficient diet along with a control group to determine the influence on nerve and cognitive performance in their offspring. They reported decreased free T4 concentrations in offspring in the marginal Iodide deficient group along with a downward trend regarding their learning and memory capacity which was more profound in the low-Iodide group¹⁰². Another investigation of a marginal ID model focusing on effects in the offspring of Long Evans rats by Gilbert et al. outlined that fetal T4 in brain and serum was reduced in a dose-dependent manner but that neonatal T4 was only reduced in serum¹⁰³. The

1.2 Disruption of TH Homeostasis and Their Consequences

detected reduction in fetal tissue was further associated with deficits in synaptic function in the hippocampus and that the adult offspring had impaired excitatory synaptic transmission¹⁰³. In concordance with the reported decrease of T4 in plasma Pedraza et al. reported this effect for young female Wistar rats receiving different Iodide-rich diet starting from day 50 of age. Examination of T3 and T4 content and DIO activity in other tissues revealed that the T3 was significant affected compared to T4. An overall increase of T3 was detected in BAT, lung and ovary and a T3 decline in the adrenal and thyroid gland¹⁰⁴. On contrary, T4 declined with decreasing Iodide concentration in every tissue investigated. DIO2 activity in brain and BAT and DIO1 activity in the thyroid gland were increased with decreasing Iodide¹⁰⁴. Besides ID also Iodide excess studies in rat altered the HPT-axis function in male offspring causing hypothyroidism and a temporary impaired learning capacity^{105, 106}. All these studies emphasize the importance of sufficient Iodide supply during pregnancy without under or over exceeding the actual requirement.

1.2.4 Enhanced TH Clearance Mediated by Hepatic Enzyme Induction

Hepatic microsomal liver enzyme induction by xenobiotics is a multi-step process in rats. The proposed process includes initially the induction of microsomal liver enzymes leading to increased hepatic clearance of T4 which is followed by decreased T4 concentration in the circulation. This causes a negative feedback to the HPT-axis answering with an elevated secretion of TSH to stimulate TH synthesis which also stimulates thyroid follicular cell proliferation leading chronically to thyroid tumor formation¹⁰⁷. However, not every liver enzyme inducer causes an increase in TSH concentration. Exposure to Phenobarbital (PB), Pregnenolone-16 α -carbonitrile (PCN), 3-Methylcholanthrene (3MC), and Aroclor 1254 (a PCB congener mixture) caused decreased free T4 concentration with increasing dosage as well as an increase of T4 UGT activity¹⁰⁷. However, only in rats exposed to PB or PCN TSH concentration were elevated which were in correspondence to increased thyroid follicular cell proliferation emphasizing the stimulating effect of TSH¹⁰⁷. Thyroid tumor formation caused by hepatic UGT-enzyme induction and the constant stimulation of TH synthesis in the thyroid gland has been evaluated as not human relevant by the European Chemicals Agency (ECHA) due to species differences¹⁰⁸. Rats have an overall higher TSH concentration in plasma which is more sensitive to further increase after disturbance of the TH homeostasis unlike in human where TSH does not remarkably increase after exposure to hepatic enzyme inducers^{41, 42}. This difference and all the exemplary mentioned differences in section 1.1.1 might be the cause of higher sensitivity towards TH homeostasis disruption in rats compared to in humans.

1.3 Regulatory Background of Thyroid Disruption

Substances affecting TH system are also known as THDs which include these substances into the class of endocrine disruptors (ED). “An endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations (WHO definition)”¹⁰⁹ and encompasses mainly MOAs in the estrogenic, androgenic, thyroid, and steroidogenic (EATS) pathways. Potential endocrine properties need to be assessed for chemicals, biocides and pesticides during authorization, and registration processes in Europe. Relevant guidance documents to identify endocrine disruptors are in place, like the Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption by the Organisation for Economic Co-operation and Development (OECD) and the Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 by the ECHA and the European Food Safety Authority (EFSA) with support from the Joint Research Centre (JRC)^{110, 111}. In December 2022 two categories for Endocrine Disruption (ED) were introduced to classify the hazard ED based on available data generated for authorization: ED HH Cat. 1 and ED HH Cat.2 (Commissions Delegated Act C(2022) 9383)¹¹². The respective classification, labelling and packaging (CLP) guidance has been updated in December 2024 (Version 5 of part 3 Health Hazard section of the guidance on the application of the CLP criteria (guidance to regulation (EC) No 1272/2008) of substances and mixtures). A classification / categorization as ED is based on the evidence-based evaluation of specific (endocrine) MOAs leading to adverse effects. More specifically, substances are to be classified as EDs, if they fulfill the following three elements: endocrine activity, adverse effect and a biological plausible link between the endocrine activity and the adverse effect¹¹³. This ultimately leads to the classification into either one of two hazard categories for ED for human health (ED HH), where Category 1 is considered to capture substances with known or presumed ED for HH based on human, animal, and/or non-animal data. The second category (ED HH Cat. 2) involves substances demonstrating evidence for suspected endocrine activity and an adverse effect, however where the data is not sufficient to classify into category 1. The guidance also states that in case sufficient evidence is available denying relevance to humans, no classification of this substance for ED HH is needed¹¹³.

According to the OECD Conceptual Framework to identify EDs, adverse (potentially endocrine-related) effects can be identified in Level 4 and 5 studies (see Table 2 below): these studies follow the test guidelines (TG) 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents), 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents), 409 (Repeated Dose

1.3 Regulatory Background of Thyroid Disruption

90-Day Oral Toxicity Study in Non-Rodents), 451 to 453 ((Combined Chronic) Toxicity & Carcinogenicity Studies), 416 (Two-Generation Reproduction Toxicity Study) or if available 443 (Extended One-Generation Reproductive Toxicity Study). The investigated adversity parameters with regard to humans and mammals usually cover thyroid gland weight and histopathology and analysis of serum TSH, T4, and T3 concentration, if the animal studies are conducted according to more recent OECD TG¹¹¹. An overview of the TGs for assessment of THD in mammalian and non-mammalian species are listed in Table 2 and were adapted from Guidance Document 150 as well as (EU) No 528/2012 and (EC) No 1107/2009^{110, 111}. In Appendix A of the latter guidance further information is given with regard to the assessment of potential THD for human health such as which evidence of species differences needs to be provided or how to investigate increase of TH metabolism in the liver¹¹¹. The analysis of THs in tissues is currently often not available but assumed to follow the observation made in serum per default.

Table 2. Enumeration of TGs and standardized test methods available for assessment of THD.

Table was adapted and modified from “Table 9 OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals, revised 2018” included in Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 by the ECHA and the EFSA with support from the JRC¹¹¹.

	Mammalian & Non-Mammalian Toxicology	
Level 1 Existing Data & Non-test Information	- Physical & chemical properties, e.g., MW reactivity, volatility, biodegradability - All available (eco)toxicological data from standardized or non-standardized tests - Read-across, chemical categories, QSARs and other in silico predictions, and ADME model predictions	
Level 2 <i>In-vitro</i> Assays	- Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding) - Other hormone receptors assays as appropriate - High-Throughput Screens	
	Mammalian Toxicology	Non-Mammalian Toxicology
Level 3 <i>In-vivo</i> Assay providing data about selected endocrine mechanism(s)/ pathway(s)		- Amphibian metamorphosis assay (AMA) (OECD TG 231) - Xenopus embryonic thyroid signaling assay (XETA) (draft OECD TG)
Level 4 <i>In-vivo</i> Assays providing data on adverse effects on endocrine relevant endpoints	- Repeated dose 28-day study (OECD TG 407) - Repeated dose 90-day study (OECD TG 408) - Pubertal development and thyroid Function assay in peripubertal male rats (PP male Assay) (US EPA TG OPPTS 890.1500)	- Fish sexual development test (FSDT) (OECD TG 234) - Larval amphibian growth & development assay (LAGDA) (OECD TG 241) - Fish early life stage (ELS) toxicity test (OECD TG 210)

1.3 Regulatory Background of Thyroid Disruption

	Mammalian & Non-Mammalian Toxicology	
	• Pubertal development and thyroid function assay in peripubertal female • Prenatal developmental toxicity study (OECD TG 414) • Combined chronic toxicity and carcinogenicity studies (OECD TG 451 to 453) • Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 421 & 422) • Developmental neurotoxicity study (OECD TG 426) • Repeated dose 90-day oral toxicity study in non-rodents (OECD TG 409)	
Level 5 <i>In-vivo</i> Assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism	• Extended one-generation reproductive toxicity study (OECD TG 443) • Two-Generation reproduction toxicity study (OECD TG 416 most recent update)	• Fish lifecycle toxicity test (FLCTT) (US EPA TG OPPTS 50.1500) • Medaka extended one-generation reproduction test (MEOGRT) (OECD TG 240) • Avian Two-generation toxicity test in the Japanese quail (ATGT) (US EPA TG OCSP 890.2100/740-C-15-003) • Zebrafish extended one-generation reproduction test (ZEOGRT) (draft OECD TG)

With regards to the aim of reducing and finding alternatives for animal studies also referred to as 3R (replace, reduce, and refine), emphasis is nowadays put on developing and validating new approach methodologies (NAM) to mainly reduce animal testing, but also to increase speed during the process of chemical hazard prediction¹¹⁴. Regarding THD, the OECD conceptual framework testing mentions at Level 2 *in-vitro* assays to test for thyroid disruption after gathering information about physico-chemical properties and data from *in-silico* methods. However, none of the mammalian *in-vitro* assays have yet been OECD validated wherefore efforts for study validation of high throughput thyroid screening assays are ongoing to assess the performance of a number of assays for disturbance of thyroid hormone function, e.g. by the EU JRC and EURL ECVAM's European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) which are enumerated in Table 3^{110, 115}. Some of the key events developed and validated were e.g. DIO1 inhibition using human liver microsomes, inhibition of MCT8, inhibition of TPO and NIS, and interaction with TTR and/or TBG¹¹⁶.

Another alternative model that experiences increasing interest is the zebrafish (*Danio rerio*) embryo model due to its rapid development, small size, optical body transparency, and general

1.3 Regulatory Background of Thyroid Disruption

cheaper handling which has been already implemented within the regulatory framework¹¹⁷. This regulatory requirement is embedded in the OECD TG 236 which describes a Fish Embryo Acute Toxicity test using zebrafish (*Danio rerio*) to examine possible acute toxicity of a chemical on embryonic stages¹¹⁸. One advantage of this model is that until 120 hours post fertilization (hpf) it is not regulated as an animal experiment which makes it suitable to investigate early developmental stages¹¹⁹. THD assessment in other organisms is currently regulatorily implemented to test in amphibians (*Xenopus laevis*) using e.g. the larval amphibian growth and development assay or the amphibian metamorphosis assay¹¹¹. THD sensitivity in zebrafish embryos has been identified by assessing various endpoints such as e.g. eye or swim bladder development, specific gene expression, TH concentrations, thyroid follicle morphology or changes in behavior as e.g. in swimming¹²⁰⁻¹²⁵. A comprehensive approach combining all these endpoints in one study and testing various THDs with different MOAs in zebrafish embryos revealed that they are sensitive towards THD exposure and that the endpoints could be linked together showing causality for ED assessment¹²⁶. Efforts are made, how and to what extent cross-species extrapolations can be made to use non-mammalian assays for human health assessments and vice versa¹²⁷.

1.3 Regulatory Background of Thyroid Disruption

Table 3. List of *in-vitro* TH-related assays from mentioned initiative by the EU-NETVAL independent of their regulatory acceptance status.

This table was adapted from the first table in section 1 of OECD report “Thyroid in vitro methods: assessment reports by the thyroid disruption methods expert group: Reports assessing the validation status of assays from the EU-NETVAL activities”¹¹⁵. Data related to assay no. 4a is included in this work.

Assay No. (Molecular Initiating Event)	Assay Title
1b (TSH receptor)	Thyrotropin-stimulating hormone (TSH) receptor activation based on cAMP measurement
2a (TPO inhibition)	Thyroid peroxidase (TPO) inhibition based on oxidation of Amplex UltraRed® (TPO-AUR)
2c (Inhibition of tyrosine iodination)	Tyrosine iodination using liquid chromatography (TYRO-IOD)
3a (Binding to serum proteins TTR and TBG)	Thyroxine-binding prealbumin (TTR) / thyroxinebinding prealbumin (TBG) binding using fluorescence displacement (ANSA). (TTR-ANSA)
3b (Binding to serum protein TTR)	Thyroxine-binding prealbumin binding using fluorescence displacement. (TTR FITC T4)
4a (Inhibition of Deiodinase 1 activity)	Deiodinase 1 activity based on Sandell-Kolthoff reaction
4b (Inhibition of THs glucuronidation)	Inhibition of THs glucuronidation using liquid chromatography/mass spectrometry
6a (TRα and TRβ receptor activation (agonist activity))	Human TH receptor alpha (TR α) and Human TH receptor beta (TR β) reporter gene transactivation measuring agonist activities (TR α and TR β reporter assays)
6b (Human TRβ receptor (in)-activation (agonist and antagonist activity))	TR CALUX human TH receptor beta (TR β) reporter gene transactivation measuring agonist and antagonist activities
7a (Alteration of intrafollicular T4-content in Zebrafish eleutheroembryos)	Measurement of intrafollicular T4 using Zebrafish eleutheroembryos (ZETA)
8a (Alteration of cell proliferation of TH responsive cells)	T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin (T SCREEN)

2 Outline

THs are essential regulators involved in several processes during development and growth as well as in metabolic pathways. Their synthesis in the thyroid gland is regulated via a complex negative feedback mechanism via the HPT-axis whereas e.g. low circulating TH concentrations induce TSH secretion to induce TH production. This complex homeostasis warrants several steps where THD could interfere with the system leading to serious adverse reactions like (e.g. weight loss, struma, tachycardia), hypothyroidism (e.g. obesity, infertility, heart disease, neurodevelopmental disorders). Exemplary THDs are industrial chemicals (PCBs, PBDEs), pesticides, or thyreostatic drugs (PTU, MMI) which bind to transport proteins, directly disturb TH synthesis by inhibiting the enzyme TPO or inhibit the metabolizing enzymes DIOs. However, a TH system disturbance is also manifested by insufficient Iodide intake which is even more crucial in pregnant women. During pregnancy maternal T4 is the sole fetal TH supply since its own thyroid gland is not active yet. As outlined above maternal ID can cause mental retardation, impaired growth and in severe cases lead to cretinism that is why developmental neurotoxicity is an endpoint of concern for regulatory substance assessment. The T-modality is currently assessed *in-vivo* in the following OECD TGs: 407, 408, 409, 416 (or 443 if available), and 451 to 453 according to the Guidance for the Identification of Endocrine Disruptors in the Context of Regulations, (EU) No 528/2012 and (EC) No 1107/2009¹¹¹. Several *in-vitro* methods assessing thyroid disruption are under OECD validation process which is summarized in the EU-NETVAL report¹¹⁵. Usually, thyroid outcome parameters assessed are majorly thyroid gland weight and histopathology and in case of assumably treatment-related thyroid effects further measurement of TSH, T4, and T3 concentrations in serum. T4 and T3 concentrations in tissue are not measured but are assumed from detected concentration in serum however correlation between the two compartments especially e.g. serum and brain are not known. This means e.g. that a substance causing TH alterations in serum does not necessarily cause an TH alteration to the same extent as detected in serum in brain. This data gap regarding TH concentration in brain is crucial when remembering maternal TH influence on offspring neurodevelopment as well as that the analysis in tissue could give additional information about metabolic processes. Before deriving statements about whether a substance causes alterations, a profound knowledge needs to be obtained focusing on biological concentration in the given matrix in this work namely rat plasma and brain. Therefore, the aim of this project was to develop and validate reliable and precise TH analysis in rat plasma and brain using an automated online-solid-phase-extraction

2 Outline

liquid chromatography tandem mass spectrometry (online-SPE-LC-MS/MS) system. The methods include multiple TH analytes relevant to the TH homeostasis such as T4, T3, rT3, T2, T1, T4-glucuronate, T4-sulfate, monoiodo-, and diiodotyrosine (MIT & DIT). The project started with the establishment of the method in rat plasma followed by rat brain. For both matrices in-house available material from dosed rat studies were analyzed and data compared to available literature. Additionally, control samples from pups on PND4 and PND21 were received to establish knowledge about the nominal TH concentrations in plasma and brain in the offspring. Brain analysis was further expanded to region-specific analysis in rat cerebellum and cortex and plasma analysis was expanded to assess free fraction of THs. These specific analyses were established to investigate TH regulation into more detail as for example into brain regional age dependency and whether free THs in plasma are more sensitive parameter towards TH alterations. Furthermore, the influence of Iodide supplementation and non-supplementation in the diet given from 4 weeks before mating until PND30 on TH concentrations was examined in parental and offspring animals. Animals from each diet group will be analyzed regarding their effects on TH concentrations in plasma and brain with focus on PND4 and 21. Another THD target are DIO enzymes which are converting THs to the less-iodinated successor. DIO1 activity was evaluated in human liver microsomes after exposure to different substances by direct analyzing rT3 and T2.

Besides TH analysis in rat tissues and human liver microsomes, the developed analytical method was further expanded to ecotoxicology relevant testing systems such as the zebrafish (*Danio rerio*). Since thyroid function is highly conserved in nature, certain factors influencing the thyroid function in one species may highly likely influence other species in a similar manner. The zebrafish has been shown to be a good predictor for neurobehavioral assessment and TPO inhibition. Therefore, first approaches were made to analyze THs in the developing zebrafish from 24 to 120 hpf. For verification of the method a positive-control exposure with PTU was investigated over four days to determine if changes in TH concentrations are detectable.

3 Materials

3.1 Thyroid Hormone Solutions

Table 4. Stock solutions of thyroid hormones.

Name	Supplier
3,3',5,5'-tetraiodo-L-thyroxine (T4)	Sigma-Aldrich St. Louis, MO 63118, USA
3,3',5-triiodo-L-thyronine (T3)	
3,3',5'-triiodo-L-thyronine (reverse T3; rT3)	
3,3'-diiodo-L-thyronine (T2)	
3-monoiodo-L-thyronine (T1)	Toronto Research Chemicals Toronto, ON, Canada, M3J 2J8
Thyroxine 4'-O-Sulfate Disodium Salt (T4-S)	
Thyroxine 4'-O-Beta-D-Glucuronide (T4-Gluc)	
3-Iodo-L-Tyrosine (Monoiodotyrosine, MIT)	Sigma-Aldrich Chemie GmbH, 89555 Steinheim, Germany
3,5-Diiodo-L-Tyrosine dihydrate	

3.2 Isotope-Labeled Standards

Table 5. Purchased isotope-labeled standards of thyroid hormones.

Name	Supplier
L-Thyroxine- ¹³ C ₆ solution (¹³ C ₆ -T4)	100 µg/mL in methanol with 0.1N NH ₃ , ampule of 1 mL, certified reference material, Cerilliant® Sigma-Aldrich Company, Round Rock, TX, USA, 78665
3,3',5-Triiodo-L-thyronine- ¹³ C ₆ solution (¹³ C ₆ -T3)	
3,3',5'-Triiodo-L-thyronine- ¹³ C ₆ solution (¹³ C ₆ -rT3)	
3, 3'-Diiodo-L-Thyronin-(phenoxy- ¹³ C ₆) (¹³ C ₆ -T2)	Sigma-Aldrich St. Louis, MO 63118, USA
3-Iodo-L-thyronine- ¹³ C ₆ (¹³ C ₆ -T1)	Toronto Research Chemicals Toronto, ON, Canada, M3J 2J8

3.3 Chemicals

Table 6. Chemical list.

Name	Supplier
Acetic Acid (AA)	100 % for analysis, ACS, ISO, Ph.Eur., EMSURE®, Merck KGaA, 64271 Darmstadt, Germany
Acetonitrile (ACN)	“LC-MS CHROMASOLV™ ≥ 99.9 %” grade Honeywell Specialty Chemicals Seelze GmbH, 30926 Seelze, Germany
Isopropanol	
Methanol (MeOH)	
Biofloat™ Clean Coating Solution	faCelliate, Chemovator GmbH, 68169 Mannheim, Germany
BupH™ Phosphate Buffered Saline (PBS) Packs	Dissolve in 500 mL Milli-pore water, Thermo Scientific, Rockford, IL 61101, USA
Dimethyl Sulfoxide (DMSO)	≥ 99.9 %, anhydrous, Sigma-Aldrich St. Louis, MO 63118, USA
Formic Acid (FA)	98-100 % for analysis, ACS, Ph.Eur., EMSURE®, Merck KGaA, 64271 Darmstadt, Germany
Millipore Water, Milli-Q® station	Merck KGaA, 64271 Darmstadt, Germany

3 Materials

3.4 Laboratory Items

Table 7. Utilized laboratory items.

Name	Supplier
0.22 µm Ultrafree-CL filter, UFC40GV	Merck Millipore; Carrigtwohill, Co Cork, Ireland
1 mL LC clear glass vial	Labsolute Th. Geyer GmbH & CoKG, 71272 Renningen, Germany
Amicon Ultra 0.5 mL filter	Merck Millipore Ltd.; Carrigtwohill, Co Cork, IRL
Centrifree® Centrifugal Filters	Merck Millipore Ltd.; Carrigtwohill, Co Cork, IRL
Eppendorf Tubes®	Safe-Lock Tubes, 2.0 mL, Eppendorf AG, 22331 Hamburg, Germany
96-well plate Oasis Mixed-Mode Cation-eXchange (MCX, 10 mg sorbent/well, 30 µm, Waters, Eschborn, Germany)	Waters GmbH, 65760 Eschborn, Germany
Oasis Online SPE hydrophilic–lipophilic balance (HLB) cartridges (10 × 1 mm & 10 x 2 mm, 30 µm)	Waters GmbH, 65760 Eschborn, Germany
Pipette Tips	Sarstedt AG& Co.KG, 51588 Nümbrecht, Germany
Pipettes	Eppendorf Research Plus, Eppendorf AG, 22331 Hamburg, Germany
Protein LoBind Tube	Safe-Lock Tubes, 2.0 mL, Eppendorf AG, 22331 Hamburg, Germany
PureProteome Albumin/IgG-Depletionskit	Merck KGaA, 64271 Darmstadt, Germany
Raptor biphenyl LC column 2.7 µm, 50mm × 2.1 mm	Restek Corporation, Bellefonte, PA 16823, USA
Sealing Tape, optically clear	Sarstedt AG& Co.KG, 51588 Nümbrecht, Germany
Silicone Micromat	Thermo Fisher Scientific, 52379 Langerwehe, Germany
Single-Use RED Plate with Inserts	Thermo Scientific, Rockford, IL 61101, USA

3.5 Technical Instruments

Table 8. List of technical devices used.

Name	Supplier
Allegra X-15R Centrifuge	Beckman Coulter GmbH, 47807 Krefeld, Germany
Bandelin Sonorex (Water Bath & Sonication), Typ RK510H	Bandelin Electronic GmbH & Co.KG, 12207 Berlin, Germany
Bead Ruptor Elite	Biolabproducts GmbH, 23816 Bebensee, Germany
Centrifuge 4K15C	Sigma Laborzentrifugen GmbH, 37520 Osterode am Harz, Germany
Eppendorf Centrifuge 5417R	Eppendorf AG, 22331 Hamburg, Germany
Freeze Dryer Gamma 2-16 LSC	Christ®, Martin Christ Gefriertrocknungsanlagen GmbH, 37520 Osterode am Harz, Germany
IR-Dancer 360 (Infra-Red Vortex Evaporator)	Hettlab, Andreas Hettich GmbH & Co. KG, 78532 Tuttlingen, Germany
Symbiosis Pharma System	Spark Holland B.V., 7812 HZ Emmen, The Netherlands
Thermomix Compact and Comfort Eppendorf	Netheler-Hinz GmbH, 22331 Hamburg
Triple Quad 5500 Mass Spectrometer (MS)	AB Sciex LLC, Framingham, MA 01701, USA
Universal 320R Centrifuge, Typ 1406	Hettich Centrifuges, Andreas Hettich GmbH & Co. KG, 78532 Tuttlingen, Germany
Vortexer-Genie 2®, Model G-560E	Scientific Industries Inc., Bohemia, N.Y. 11716, USA

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3.6 Standard Solutions

From the listed stock in caption 3.1 and 3.2 stock solutions were prepared in dimethyl sulfoxide (DMSO) for the analytes which were not purchased as liquids. In a second step serial dilutions were prepared from the stock solutions to prepare standard solutions adjusted to the concentration needed for calibration. These standard solutions were generated in 70 % aqueous methanol (MeOH). Plasma serial dilutions ranged from steps 0.004, 0.008, 0.015, 0.03, 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10, 20, to 30 ng/mL and brain serial dilutions from 0.005, 0.01, 0.02, 0.05, 0.075, 0.15, 0.3, 0.5, 1, 2 to 4 ng/mL. The calibration ranges for each analyte in each matrix were fitted for the expected concentration in the samples.

3.7 Animal Samples

Rat brain and plasma samples were received from mostly untreated Wistar rats (CrI:WI(Han), control group) from different in-house studies conducted over the years (Table 9, Table 10 & Table 12). The Wistar rats were held under standardized conditions meaning 12 hours light and dark shifts at 20 to 24°C, 45–65 % humidity and fifteenfold air changes per hour. Food and drinking water were available *ad libitum* which both were examined regularly for possible contaminants. The rats were housed in an AAALAC accredited animal facility and in accordance with the German Animal Welfare act. The studies have been authorized by the local supervising authority Landesuntersuchungsamt Rheinland-Pfalz, Germany (approval number 23 177-07/G 17-3-080, 23 177-07/G 22-3-050, 23 177-07/G 13-3-016 and 23 177-07/G 12-3-018). In studies including pups, the pups underwent daily observation for clinical symptoms. Some of the samples analyzed were not obtained from recently conducted studies and they were stored since sampling in sealed vessels under an inert gas atmosphere at –80 °C. Internal long-term storage stability studies of 16 compounds measured over six different timepoints have shown that storage is not an influencing factor over time¹²⁸. Same standardized conditions were kept for the conducted non-supplemented Iodide diet study in Wistar rats (CrI:WI(Han)) except that the control group received *ad libitum* mouse and rat maintenance diet ("GLP", Granovit AG, Kaiseraugst, Switzerland) and the dosage group special diet which was not supplemented with Iodide (2036.PH.A05 Mouse/rat modified Iodide free Granovit AG, Kaiseraugst, Switzerland). Administration of the diet begun four weeks prior to mating and continued until PND30. Blood sampling took place in the parent animals on days 7, 14 and 28 prior to mating and in offspring on PND4, 21, and 30 from which additionally brain samples were retrieved tabularized in Table 11. Neurobehavior testing was conducted in subsets of offspring on PND22

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and PND25 which consisted of motor activity testing and the Morris water maze test which results are not reported since they were out of scope of this work.

Table 9. Overview of control rat samples received, and measured data shown in 5.6.

Study Year	Study No.	Age	Matrix	Amount of Female Control Animals (Sampling Day)	Amount of Male Control Animals (Sampling Day)
2015	1	97-99d	Plasma	6, 10 & 6 (7, 14, & 28)	10, 9 & 10 (7, 14, & 28)
		97-99d	Brain	10 (28 d)	10 (28)
	2	97-99d	Plasma	10, 10 & 9 (7, 14, & 28)	8, 8 & 10 (7, 14, & 28)
		97-99d	Brain	7 (28)	10 (28)
2022	3	Dams	Plasma	10 & 10 (PND4 & 21)	/
			Brain		
		PND4	Plasma	10	10
			Brain	10	10
		PND21	Plasma	10	10
			Brain	10	10
2023	4	PND4	Plasma	5	1
			Brain	6	1
		PND21	Plasma	10	10
			Brain	10	10
2023	5	71-72d	Whole-Brain	5	5
			Cerebellum	5	5
			Cortex	4	5
2023	6	PND4	Cerebellum	6	6
			Cortex	6	6
		PND22	Cerebellum	6	6
			Cortex	6	6
2024	7	PND4	Plasma	1	2
			Whole-Brain	5	5
			Cerebellum	4	4
			Cortex	4	4
		PND21	Plasma	10	10
			Whole-Brain	5	5
			Cerebellum	4	4
			Cortex	4	4

Table 10. List of received samples which were exposed to the mentioned substance.

Results of TH analysis in plasma are shown in section 5.7. For calculation from ppm to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d was assumed. LD refers to low dose and HD to high dose.

Study Year	Substance	Age Start [days]	Dosage Group	Dose [mg/kg bw/d]	Number of Female Animals (Sampling Day)	Number of Male Animals (Sampling Day)
2005	PTU	69-71	Control	0	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
			LD	1	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	10	5, 5, & 5 (7, 14, & 28)	4, 5, & 5 (7, 14, & 28)
2006	Methimazole	69-71	Control	0	10, 9 & 0 (7, 14, & 28)	10, 9 & 0 (7, 14, & 28)
			LD	25	4, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	100	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)

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Study Year	Substance	Age Start [days]	Dosage Group	Dose [mg/kg bw/d]	Number of Female Animals (Sampling Day)	Number of Male Animals (Sampling Day)
2007	Ethylene Thiourea	69-71	Control	0	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
			LD	6 (75 ppm)	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	24 (300 ppm)	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
2007	Aroclor 1254	70-72	Control	0	5, 5, & 5 (7, 14, & 28)	3, 5, & 5 (7, 14, & 28)
			LD	5	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	25	5, 5, & 5 (7, 14, & 28)	4, 5, & 5 (7, 14, & 28)
2011	Vinclozolin	69-71	Control	0	4, 5, & 4 (7, 14, & 28)	5, 3, & 4 (7, 14, & 28)
			LD	80 (1000 ppm)	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	240 (3000 ppm)	5, 5, & 5 (7, 14, & 28)	4, 5, & 5 (7, 14, & 28)
2007	Aroclor 1254	70-72	Control	0	5, 5, & 5 (7, 14, & 28)	3, 5, & 5 (7, 14, & 28)
			LD	5	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	25	5, 5, & 5 (7, 14, & 28)	4, 5, & 5 (7, 14, & 28)
2024	3,3',5,5'-Tetrabromo-bisphenol A	About 70	Control	0	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
			LD	300	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	1000	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
	Perflouro-hexane-sulphonic Acid		Control	0	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
			LD	6	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	20	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
	Phenyl Isothiocyanate		Control	0	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
			LD	15	5, 5, & 5 (7, 14, & 28)	5, 5, & 5 (7, 14, & 28)
			HD	30 (7d) 22.5 (28d)	5, 0, & 3 (7, 14, & 28)	5, 0, & 4 (7, 14, & 28)

3 Materials

Table 11. Animal samples from study receiving different amounts of Iodide in diet.

Study Year	Diet	Age	Matrix	Number of Female Animals (Sampling Day)	Number of Male Animals (Sampling Day)
2024-2025	Supplemented (S)	70 d	Plasma	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
		PND4	Plasma	6	1
			Whole-Brain	11	1
		PND21	Plasma	7	10
			Whole-Brain	7	10
		PND30	Plasma	10	10
			Whole-Brain	10	10
	Non-Supplemented (NS)	70 d	Plasma	10, 10 & 10 (7, 14, & 28)	10, 10 & 10 (7, 14, & 28)
		PND4	Plasma	2	5
			Whole-Brain	10	12
		PND21	Plasma	10	10
			Whole-Brain	10	10
		PND30	Plasma	10	9
			Whole-Brain	10	9

Table 12. Tabular summary of analyzed samples from studies with masked substances and their given concentration.

For calculation from ppm to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d was assumed.

	Study 1	Study 2	Study 3	Study 4
Substances	I G A J E H K D	C F	B	I C G
Dose [mg/kg bw/d]	10 25 12 (150 ppm) 4.8 (60 ppm) 5.2 (65 ppm) 5 32 (400 ppm) 0.15	150 0.4	32 & 64 (400 & 800 ppm)	3 & 10 50 & 150 8 & 25
Sampling day [d] – Plasma	7	7	7, 14 & 28	7, 14 & 28
Sampling day [d] – Brain	7	7	28	28
Administration	gavage or in diet	gavage	diet	gavage
Age at beginning of administration period [d]	69-72			
Number of animals per dosage group	5	10	5	5

4 Methods

4.1 Sample Preparation

4.1.1 Total THs in Rat Plasma

A volume of 50 μL rat plasma was used to analyze total thyroid hormone concentration. Before protein precipitation with 200 μL 1 % formic acid (FA) in MeOH, 20 μL of the internal standard mix was added. Final concentration of the isotope-labeled THs were 0.05 ng/mL $^{13}\text{C}_6$ -T1, 0.1 ng/mL $^{13}\text{C}_6$ -T2, -T3, and -rT3 and 5 ng/mL $^{13}\text{C}_6$ -T4. The protein precipitate mixture was incubated for 30 min at $-23\text{ }^{\circ}\text{C}$ ($\pm 3\text{ }^{\circ}\text{C}$) and further vortexed after 15 min. Prior to pipetting the sample into an Amicon Ultra 0.5 mL filter (Merck Millipore; Carrigtwohill, Co Cork, IRL), the samples were vortexed a second time. The Eppendorf vial as well as the used pipette tip were kept from each sample after loading it onto the filter and were consecutively washed twice with 150 μL 70 % aqueous MeOH. The centrifugation of the samples after protein precipitation was at 14,000 rpm for 30 min at $4\text{ }^{\circ}\text{C}$ (Eppendorf Centrifuge 5417R, Eppendorf AG, 22331 Hamburg, Germany) and the following centrifugation after each wash step were at 14,000 rpm for 15 min at $20\text{ }^{\circ}\text{C}$. The eluate was transferred into a 1 mL LC clear glass vial (Labsolute Th. Geyer GmbH & CoKG, Renningen, Germany), and evaporated to dryness at $40\text{ }^{\circ}\text{C}$ using an Infra-Red Vortex Evaporator (IR-Dancer 360, Hettlab, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The dried pellet was resuspended in 98 μL MeOH, sonicated for around 5 min and 42 μL Milli-Q water was added to reach a final volume of 140 μL . Prior to analysis the LC glass vials were sealed using a silicone Micromat (Thermo Fisher Scientific, Langerwehe, Germany).

4.1.2 Total THs in Rat Brain

Rat brains were lyophilized to ensure an improved homogenization of the tissue by pulverization (Bead Ruptor Elite, biolab products, Bebensee, Germany). To proceed with sample preparation a definite amount of pulverized brain was weighed-in depending on the age and brain region of the given rat which is listed in Table 13. After adding 300 μL phosphate-buffered saline (PBS) to the weighed-in brain samples, they were sonicated for 5 min. Then 20 μL of the internal standard mixed was pipetted and mixed at 1,400 rpm for 10 min. The final concentrations of the $^{13}\text{C}_6$ -labeled internal standards were 0.1 ng/mL $^{13}\text{C}_6$ -T1, -T2, and- rT3, 0.4 ng/mL $^{13}\text{C}_6$ -T3 and 0.5 ng/mL $^{13}\text{C}_6$ -T4. The following protein precipitation was conducted by adding 900 μL 1 % FA in acetonitrile (ACN) and continued as described above except that instead of using an Amicon Ultra 0.5 mL filter, a 0.22 μm Ultrafree-CL filter (UFC40GV,

4.1 Sample Preparation

Merck Millipore; Carrigtwohill, Co Cork, Ireland) was utilized and the centrifugation times were 15 min at 5,100 rpm at 4 °C and 6 min and 10 min at 5,100 rpm at 20 °C (Centrifuge 4K15C, Sigma Laborzentrifugen GmbH, 37520 Osterode am Harz, Germany). Additionally, the washing steps involved 200 µL 80 % aqueous ACN. Due to the high volume at the end of this procedure (≥ 1 mL) a two-times evaporation was needed.

Table 13. Amount of weighed-in brain per age and per analyzed brain region.

Age	Brain Region	Amount [mg]
PND4	Cerebellum	all
	Cortex	7
	Whole-Brain	7
PND21	Cerebellum	10
	Cortex	20
	Whole-Brain	20
Adult	Cerebellum	20
	Cortex	40
	Whole-Brain	40

4.1.3 Total THs in Formalin-Fixed and Paraffin-Embedded Rat Brain

A first test trial to extract THs from formalin-fixed (FF) and paraffin-embedded (PE) half-brain from adult rats (n=2) was established. From each rat brain one half was prepared with a different tissue fixation meaning either embedded in paraffin or fixed in formalin.

First testing of preparation regarding the formalin-fixed half-brain consisted only of washing it in Millipore water prior to freezing at -80 °C. On contrary, the PE half-brain was placed into a beaker with hot water (~ 70 - 80 °C) to melt the paraffin in which it was embedded as four pieces. After around 10 min the brain pieces were transferred into a second beaker with hot water using forceps to wash off remaining paraffin residues. This washing step was repeated two more times prior to freezing it at -80 °C. Both tissue preparations were lyophilized and afterwards pulverized (Bead Ruptor Elite, biolab products, Bebensee, Germany). Easy pulverization was achieved regarding the FF tissue, however PE half-brain did not yield a homogenous pulverization. Thus, 30 mg of brain pieces were weighed whereas 20 mg of FF-powder was weighed in for analysis. The subsequent sample preparation was conducted as described under 4.1.2.

For the second testing the procedure was modified as follows. FF half-brain was washed twice with 100 % Ethanol (EtOH) for around 5 min after which it was placed into a flask with PBS under mixing at 150 rpm. Every 2 h PBS solution was replaced and after 6 h PBS was exchanged with Millipore H₂O in which it was incubated overnight gentle mixing at 150 rpm. The second half-brain embedded in paraffin was initially handled the same way as described for the first procedure. After washing in hot water, the four half-brain pieces were immersed in a descending EtOH series from 100, 95, 85 to 75 % (v/v). Each series step was performed twice

4.1 Sample Preparation

and each time for 5 min. Ultimately, the half-brain pieces were washed in Millipore H₂O and incubated overnight whereas during the day Millipore H₂O was exchanged around every 2 h. The next day the tissues were prepared for lyophilization, weighed-in and prepared for analysis as described under 4.1.2.

4.1.4 rT3 and T2 in Human Liver Microsomes

The estimation of DIO1 inhibition was assessed using human liver microsomes. This assessment was done in cooperation with a laboratory in the department of “Experimental Toxicology and Ecology” at BASF SE in Ludwigshafen which have previously published the validation of the DIO1 assay using the Sandell-Kolthoff (SK) reaction as readout parameter^{129, 130}. Prior to sample preparation, the samples were diluted 1:400 in two dilution steps because of the high concentration of substrate rT3 (5 μ M) used in the assay. From the final dilution 50 μ L was taken, 20 μ L of isotope-labeled standard mix added and prepared as described in section 4.1.1¹³¹.

4.1.5 Free THs in Rat Plasma

During method development to evaluate free TH (fTH) fraction in rat plasma, two different method approaches were tested. At the beginning, physiological spiked PBS was used to assess recovery during development. PBS was used because no TH free rat plasma was available which could function as an analyte free matrix but also due to the high volume needed and to the limited availability of rat plasma samples, an analysis of spiked rat plasma compared to non-spiked plasma was not possible.

4.1.5.1 Ultrafiltration (UF)

For ultrafiltration (UF) Centrifree® centrifugal filters (Merck Millipore Ltd., Carrigtwohill, Ireland) were utilized because it has been shown that they exhibit better performance than other UF devices¹³². Plasma vials were equilibrated to 37 °C for 15 min and a plasma volume of 200 μ L or 300 μ L was added onto the device. The samples were centrifuged through a 30 kDa membrane at 2539 rpm for 30 min at 37 °C (Allegra X-15R Centrifuge, Beckman Coulter GmbH, 47807 Krefeld, Germany). The ultrafiltrate was transferred into a LC glass vial to which the internal standard mix was added and evaporated to dryness. Like the other samples, the dried pellet was resuspended in 140 μ L aqueous MeOH.

4.1.5.2 Rapid Equilibrium Dialysis (RED)

Rapid equilibrium dialysis (RED) was conducted with Pierce™ RED Device Single-Use Plate with Inserts (Thermo fisher scientific, Illinois 61101, USA) and a membrane molecular weight

4.1 Sample Preparation

cut-off (MWCO) of 8 kDa. The plasma sample (200 or 300 μL) was pipetted into the sample chamber (surrounded by the membrane) and 400 or 550 μL PBS (100 mM Sodium Phosphate and 150 mM Sodium Chloride) into the buffer chamber. The plates were incubated for either 6 h or 16 h to reach equilibrium of the fTH fraction. A volume of ≥ 180 μL was removed from each chamber into separate LC glass vials except the extracted volume out of the sample chamber which was added into an Eppendorf tube for protein precipitation (see 4.1.1). All samples received isotope-labeled standard mix, were evaporated, and resuspended with 140 μL aqueous MeOH. The volume taken out of the buffer chamber is analyzed regarding the fTHs concentration in the given sample.

4.1.6 TSH in Rat Plasma

The TSH method development is not terminated yet, therefore the current status of sample preparation is subsequently described. To remove the high abundant albumin in rat plasma a PureProteome™ Albumin/IgG Depletion Kit (Merck KGaA, Darmstadt, Germany) was used. Therefore, a working solution of 1 x PBS (stock solution 10 x PBS) was prepared and 950 μL of the magnetic bead solution was inserted into a 2 mL Protein LoBind Eppendorf tube (Eppendorf AG, Hamburg, Germany). The magnetic beads were attracted magnetically towards the bottom of the sample tube from which the storage solution was removed. This procedure was repeated twice using 500 μL 1 x PBS to wash the magnetic beads. In parallel, the rat plasma sample was diluted in 1 x PBS to a final volume of 100 μL (1:4, 25 μL plasma & 75 μL 1 x PBS). The diluted plasma sample was added to the washed magnetic beads and then incubated for 60 min under continuous mixing at room temperature (RT). The depleted plasma was separated magnetically from the beads and saved in a separated sample tube. As a final step the magnetic beads were washed three times with 500 μL 1 x PBS whereas the wash fractions were combined with the depleted plasma. The obtained plasma underwent a standardized reduction, alkylation, and protein digestion protocol for which lastly the sample was incubated with a Trypsin/Lys-C Mix overnight at 37 °C. The next day the reaction was stopped with FA (final 0.5-1 %). As a last cleanup the digested plasma samples were purified using a 96-well plate Oasis Mixed-Mode Cation-eXchange (MCX, 10 mg sorbent/well, 30 μm , Waters, Eschborn, Germany) offline SPE cartridge plate. First, the wells were conditioned using 500 μL of a ACN/H₂O/25 % ammonium hydroxide solution (15:4:1, v/v/v), washed with 500 μL 100 % MeOH, further washed with 500 μL 0.1 % FA in H₂O (v/v) after which the entire sample was added into the well and again washed twice with 500 μL MeOH/H₂O/FA (80:20:1, v/v/v). After each of these steps the 96-well plate was centrifuged for 2 min at 2000 rpm at 4 °C utilizing the slowest acceleration (Universal 320R Centrifuge, Typ 1406, Hettich Centrifuges, Andreas

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Hettich GmbH & Co. KG, 78532 Tuttlingen, Germany) and the eluate was collected in a waste plate which was discarded. Ultimately, three elution steps were conducted each time with 150 μ L ACN/H₂O/25 % ammonium hydroxide solution (15:4:1, v/v/v) whereas prior to centrifugation the well was incubated with the solution for around 1 to 2 min at RT and the subsequent eluates were combined. The collected eluates were evaporated until dryness at 40 °C.

4.1.7 TH Analysis in Whole Zebrafish Embryos

The TH analysis in whole zebrafish embryo was done in cooperation with a laboratory in the department of “Experimental Toxicology and Ecology” at BASF SE in Ludwigshafen who conducted the zebrafish embryo exposure and thus provided the zebrafish embryo samples. The pooled zebrafish embryos (n=50) were transferred from the delivered vial into a 2 mL Eppendorf plastic vial by adding 1 mL 80:20 (w/w) Isopropanol: H₂O to facilitate the transfer as well as ensure extraction. Further the isotope-labelled standard mix (20 μ L) and one 3 mm steel ball were inserted into the solution. Homogenization and extraction of the sample was performed on a Bead Ruptor Elite for 3 x 30 sec at 4.85 m/s (biolab products, Bebensee, Germany) as previously stated for rat brain tissue. Afterwards, the steel ball was removed, the solution centrifuged for 10 min at 12,000 rpm at 15 °C (Eppendorf Centrifuge 5417R, Eppendorf AG, 22331 Hamburg, Germany) followed by removal of the supernatant into a LC glass vial for evaporation to dryness. As in all above sample preparation descriptions, the pellet was resuspended in first 98 μ L MeOH, then sonicated and lastly 42 μ L Milli-Q water was pipetted to reach a final volume of 140 μ L.

4.2 Instrumentation

4.2.1 Online Solid-Phase Extraction (online-SPE)

After sample preparation described above, additional clean-up was provided through online SPE using a Symbiosis Pharma System (referred to as Spark, Spark Holland, Emmen, The Netherlands). The cartridges selected were Oasis™ on-line SPE hydrophilic–lipophilic balance (HLB) cartridges (10 x 1 mm & 10 x 2 mm, 30 μ m, Waters, Eschborn, Germany) due to their ability to capture all analytes. A slightly bigger cartridge was used for TH analysis in rat brain because of the higher lipophilicity of the matrix for which a few parameters regarding the SPE procedure had to be changed. The SPE procedures for both cartridge sizes are depicted in Figure 3. One of the main parameter changes were the higher wash volume as well as the increased wash volume flow rate. The high-pressure dispenser two (HPD2) focusing mode was used for complete elution of all analytes from the cartridge. The TH analytes reflect a broad range from

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less to strong lipophilic properties, therefore it was not possible to elute all analytes from the cartridge by a liquid chromatography (LC) gradient sufficiently and in a short time. The HPD2 focusing mode elutes all TH analytes from the cartridge because of elution with 100 % MeOH. After passing through the cartridge, the analytes encounter the LC flow at 100 % 0.1 % acetic acid (AA) in H₂O transferring them onto the LC column.

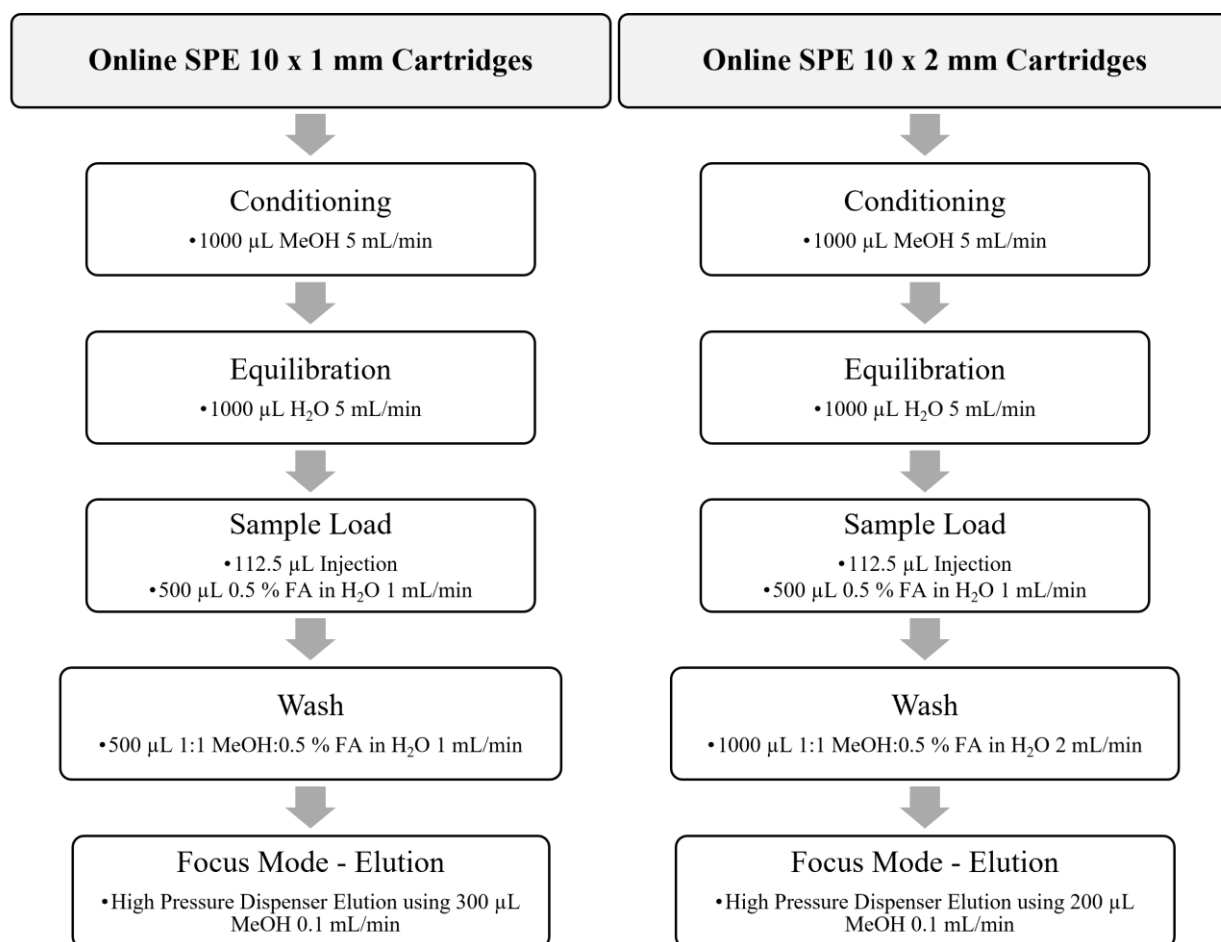


Figure 3. Schematic SPE procedure utilizing Oasis™ online SPE HLB cartridges in sizes 10 x 1 mm and 10 x 2 mm.

4.2.2 Liquid Chromatography (LC)

After HPD2 elution, the TH analytes are timely separated on a Raptor biphenyl column (2.7 µm, 50mm × 2.1 mm, Restek, Bellefonte, PA, USA). The Raptor biphenyl column was chosen because of the π - π -interaction between the aromatic rings which captured the broader hydro-, lipophilic range of the given TH analytes compared to a C18-column. As mobile phases (MP) 0.1 % AA in H₂O (MP-A) and 0.1 % AA in MeOH (MP-B) were chosen because it showed improved peak intensity. Additionally, it has been reported that ACN suppresses π - π -interaction supplied by the biphenyl column¹³³. The LC pump flow rate during HPD2 elution was set at 0.1 mL/min 100 % MP-A which increased after elution to the final flow rate of 0.3 mL/min and the initial gradient of 95 % MP-A. The LC gradients are listed in Table 14 and

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Table 15. The differences between the LC gradients occurred because of less analytes (T1, T2, rT3, T3 & T4) incorporated during rat brain analysis compared to analysis in plasma (MIT, DIT, T1, T2, rT3, T3, T4, T4-Gluc, and T4-SO₄). Furthermore, during brain method development a Spark instrument exchange took place due to maintenance of the initial instrument. This exchange caused inter alia the shorter HPD2 elution time (performance differences between Spark instruments) which consecutively led to a shorter LC run.

Table 14. LC gradient used for the analysis of plasma samples.

Time [00:00 min]	MP-A [%]	MP-B [%]
0:001	100	0
03:00	100	0
03:30	95	5
05:00	60	40
10:00	30	70
10:30	0	100
12:30	0	100
13:30	100	0
16:00	100	0

Table 15. Time and MP composition during LC gradient for TH analysis of brain samples.

Time [00:00 min]	MP-A [%]	MP-B [%]
0:001	100	0
02:00	100	0
02:30	95	5
03:30	50	50
08:30	30	70
09:00	0	100
11:30	0	100
12:30	100	0
15:00	100	0

4.2.3 Mass Spectrometry (MS)

TH analysis was performed on a Triple Quad 5500 Mass Spectrometer (MS, AB Sciex Instruments, Framingham, MA, USA). It was operated in positive electrospray ionization mode using multiple reaction monitoring (MRM). MS parameters were first adapted from previous measurements which were used for plasma analysis and in the course of method development focusing on brain matrix systemically optimized. The initial (plasma) and later adjusted general MS parameters (brain) are recorded in Table 16.

Table 16. Enumeration of general MS parameters used for plasma and brain method.

Method/Parameters	Curtain Gas (CUR)	Collision Gas (CAD)	Ion Spray Voltage (IS)	Temperature (TEM)	Ion Source Gas 1 (GS1)	Ion Source Gas 2 (GS2)
Plasma	40	11	5500	600	60	70
Brain	50	8	5500	600	50	55

4.3 Method Validation

For both matrices a method validation was accomplished according to an internal standard working procedure. Reference criteria were adopted from chapter 3 “Method validation parameters” in the Directorate-General for Health and Food Safety (DG SANTE) guidance document “SANTE/2020/12830”¹³⁴. The purpose is to show that the selected analytes can be determined with sufficient precision, specificity, and robustness in the given matrix. An alternative for plasma matrix is the usage of PBS for the preparation of the standard curve because of its physiological similarity in osmolarity and ion concentrations, as well as its buffering capacity. With regards to the brain method development preparation of the standard curve was switched to 70 % MeOH because it reflects the resuspension solvent after evaporation of the samples. Both methods were validated regarding the parameters specificity and selectivity, matrix effect, carry-over, linearity, limit of quantification (LOQ) and limit of detection (LOD).

4.3.1 Linearity

In each measurement standard curves were included in duplicates ($n=2$) at the beginning and end of each acquisition batch. Since T4 has a much higher expected concentration in plasma compared to the remaining analytes, its standard curve was prepared without the other analytes at a certain concentration. This was not true for brain analysis because this matrix yielded similar concentrations between the analytes. Each standard curve ranged from the LOD to the highest expectable concentration. Accuracy and precision at each concentration of the standard curve was calculated from the LOQ to the highest expected concentration. Therefore, accuracies should reach 85-115 % except at LOQ level set to 80-120 %. During evaluation the standard curve was plotted by area ratio of the analyte and internal standard for each point on the y-axis and on the x-axis the concentration ratio of the analyte and the internal standard. Additionally, $1/x$ weighted and the coefficient of determination R^2 was established. The criterion is hereby to be fulfilled R^2 to be ≥ 0.99 . Unknown concentration was calculated for each individual analyte by solving for x via linear regression.

4.3.2 Carry-Over

The analytes should not be carried from sample to sample since it would falsify the result. To investigate for this criterion two PBS blanks were injected following the highest concentration of the standard curve and assessed regarding detectable analytes signal. Only the signal in the first injected PBS blank was verified and the signal should not exceed 30 % of the analytes signal at LOQ level.

4.4 Data and Statistical Analysis

4.3.3 LOD and LOQ (Accuracy and Precision)

The LOD is defined as 30 % of the LOQ and it is the lowest concentration in the standard curve. The LOQ is the lowest quantifiable concentration in the standard curve and accuracy and precision of each method was verified at its LOQ concentration as well as 10 x LOQ. Five technical replicates at LOQ and 10 x LOQ level were prepared and quantified for the recovery of the added concentration. Precision was defined as the relative standard deviation (RSD). RSD is calculated through the division of the standard deviation by the mean and lastly times 100 to reach the final unit in percentage. The implemented accuracy/recovery range is 70 to 120 % with a precision smaller than 20 %.

4.3.4 Specificity and Selectivity

Specificity and selectivity examine the analytes quantification without possible disturbances within the matrix. To do so one solvent blank as well as two PBS blanks were measured and quantified regarding possible analyte peaks. The analytes area found in each blank is evaluated to the mean analytes area at LOQ level and reported as percentage [%]. The analytes signal found in each blank should not exceed 30 % of the analytes signal at LOQ level.

4.3.5 Matrix Effect

Influential effect of the matrix onto sample analysis was estimated by comparing the signal of a sample in solvent and matrix to another. As both matrices naturally contain THs a comparison of e.g., T4 in a solvent to a matrix sample is not possible. To overcome this problem the signals of the isotope-labeled TH in these samples were compared. A matrix effect is present when the signals deviate ± 20 %.

4.4 Data and Statistical Analysis

Acquisition data of each measurement was evaluated using MultiQuant Software Version 3.0.3 (AB Sciex LLC, Framingham, MA, USA). GraphPad Prism software version 8.0.2 (GraphPad Software, San Diego, CA, USA) was applied to do statistical and graphical evaluation of the data. The One-way ANOVA (Brown-Forsythe and Welch ANOVA) using unpaired t with Welch's correction was performed to determine statistical significance with a critical p value set to 0.05.

5 Results

5.1 MS Optimization of Analyte Peak Intensities

Mass Spectrometry (MS) optimization was conducted prior method validation to improve peak intensity. Duplicates of 5 mg adult rat brain were prepared according to section 4.1.2 except that they were resuspended in a total volume of 950 μ L 70 % MeOH. From this diluted brain matrix 1 ng/mL standard samples were prepared for measurement. The optimization was done sequentially starting with the parameter Curtain Gas (CUR) followed by Ion Spray, Temperature, Gas 1 (GS1), Gas 2 (GS2), and Collisional Activated Dissociation (CAD). Analyte and isotope-labeled internal standard peak heights of their most prominent mass fragments were compared individually between the values tested for the given parameter. The tested value of every parameter resulting in the most intense peak were selected to proceed with during optimization procedure. These selected values are additionally highlighted as a blue star in each figure. Besides that, final values were written in red script in the text fields below each graph's legend. Figure 4 contains results for the optimization of the first parameters; "CUR", "Ionspray", and "Temperature". As indicated by the blue star in the graphs the final selected values were 50, 5500 V, and 600 °C respectively since the highest peak intensities were achieved for all mass fragments. MS parameter optimization was continued with GS1, GS2, and CAD as seen in Figure 5. Test injections for the last two tested GS2 values (40 and 55) and CAD optimization were prepared in PBS instead of diluted brain matrix due to finishing of the diluted matrix. The final selected GS2 value of 55 was assessed by test injections prepared in diluted brain matrix and PBS. Lastly to gain a reference value for ongoing optimization using test injections in PBS. Further optimization was achieved for GS1 with a value of 50 instead of the initial 60 and for CAD of 8 instead of 11. Overall, the optimized MS parameters were CUR 50, Ion Spray 5500 V, Temperature 600 °C, GS1 50, GS2 55 and CAD 8.

Besides that, and as mentioned in method section 4.1.6 experiments were conducted to analyze TSH in rat plasma. Various sample preparation procedures were tested such as at the PureProteome™ Albumin/IgG Depletion Kit were introduced to remove the high abundant albumin in rat plasma. The aim was to remove the concentration of albumin to increase potential detection of the low concentrated TSH which was still not detected. In literature concentration of TSH in rats has been estimated for example around 1.8 ng/mL in 16 week old rats and around 2 ng/mL in pregnant and lactating rats which were typically evaluated with immunoassays such as radioimmunoassay (RIA)^{103, 135}. Conclusively, method development was not sufficient to detect TSH in rat plasma.

5.1 MS Optimization of Analyte Peak Intensities

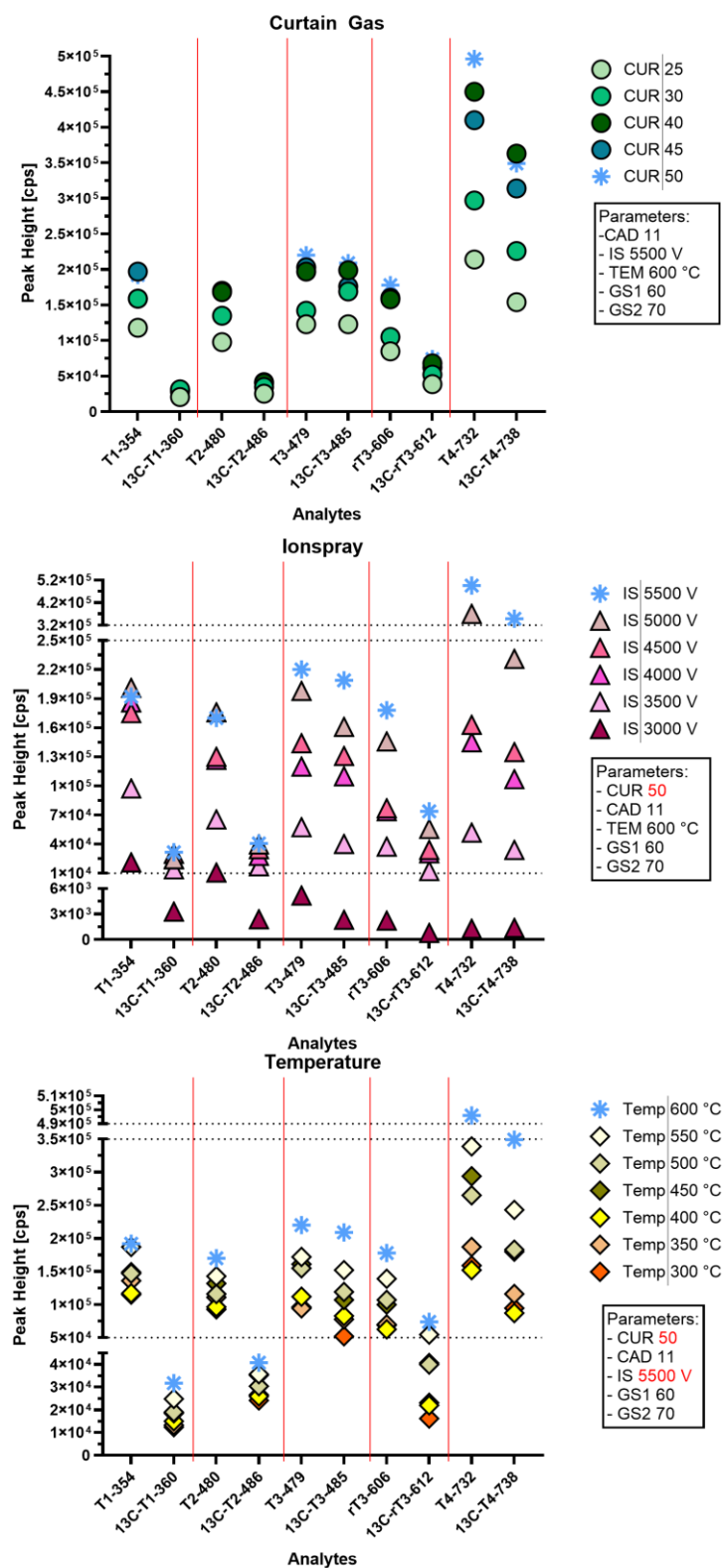


Figure 4. Procedure of MS optimization for parameters Curtain Gas (CUR), Ionspray, and Temperature. The x-axis lists pairwise the analyte and its isotope-labeled internal standard (TH & $^{13}\text{C}_6$ -TH) to the detected peak height [cps] at the given parameter setting on the y-axis. The tested parameters are depicted as differentially colored symbols, and a blue star highlights the ultimately selected parameter value in each graph. Furthermore, the text field next to the graphs lists the remaining parameter values and those already optimized are written in red. The optimization for the first parameters were “Curtain Gas”, “Ionspray”, and ended with parameter “Temperature” which values were selected to be 50, 5500 v, and 600 °C accordingly.

5.1 MS Optimization of Analyte Peak Intensities

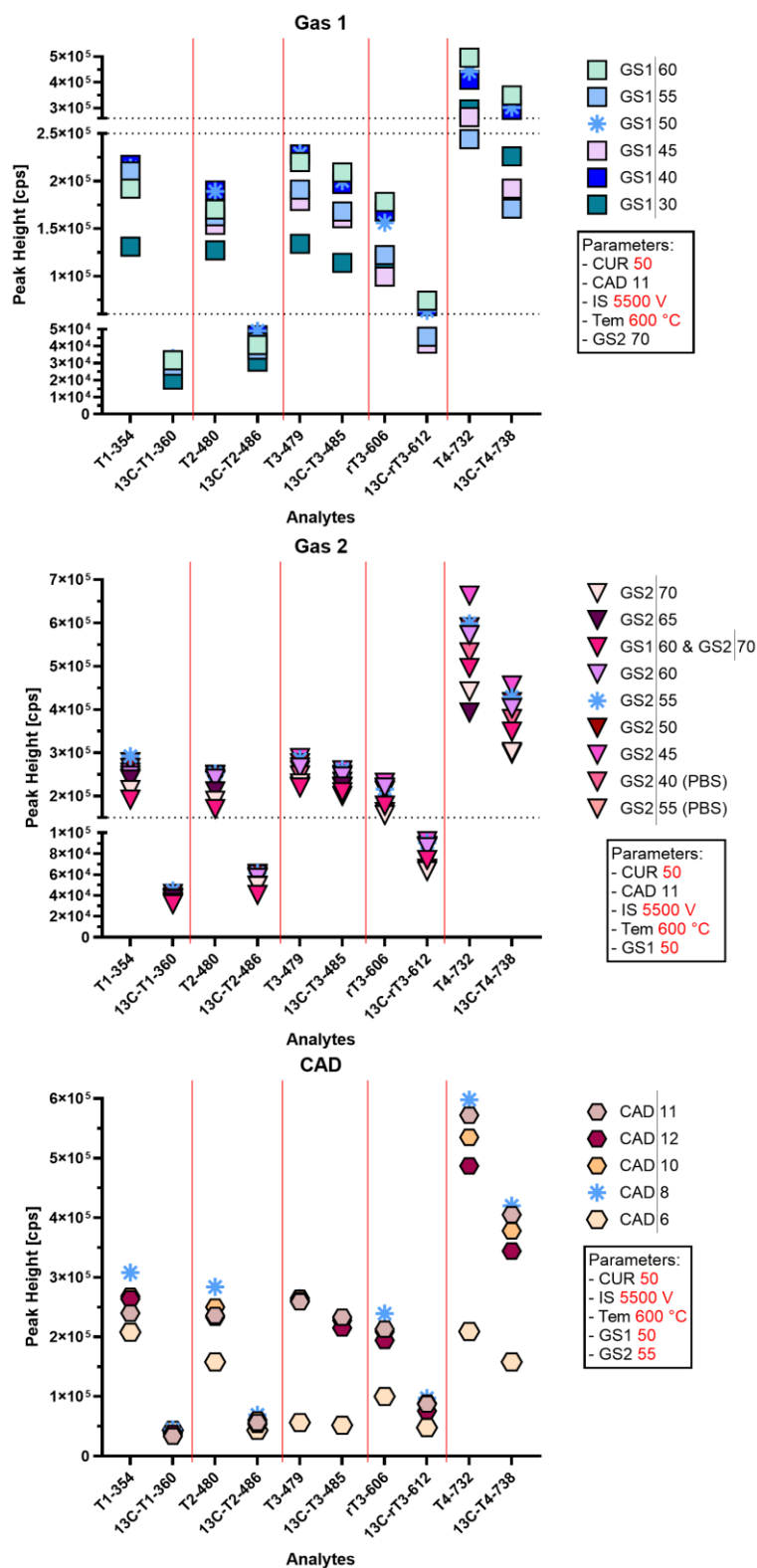


Figure 5. Second part of MS optimization for parameters Gas 1 (GS1), Gas 2 (GS2), and Collisional Activated Dissociation (CAD).

The x-axis lists pairwise the analyte and its isotope-labeled internal standard (TH & $^{13}\text{C}_6$ -TH) to the detected peak height [cps] at the given parameter setting on the y-axis. The tested parameters are depicted as differentially colored symbols, and a blue star highlights the ultimately selected parameter value in each graph. Furthermore, the text field next to the graphs lists the remaining parameter values and those already optimized are written in red. The optimized values for parameters “GS1”, “GS2”, and “CAD” were determined to be 50, 55, and 8.

5.1 MS Optimization of Analyte Peak Intensities

After completing general MS optimization, collision energy (CE) for every mass fragment was optimized. Consequently, steps of 2 V or 5 V were added as well as subtracted from initial CE values and the resulting peak height intensities were compared. The results are graphically illustrated per analyte and its corresponding isotope-labeled internal standard in Figure 6 and Figure 7. In the legend the initial CE is recorded next to the name of every symbol. For all investigated mass fragments an increase in peak height intensity was detected by adding 2 V to the initial CE values, thus this setting was chosen as the final value.

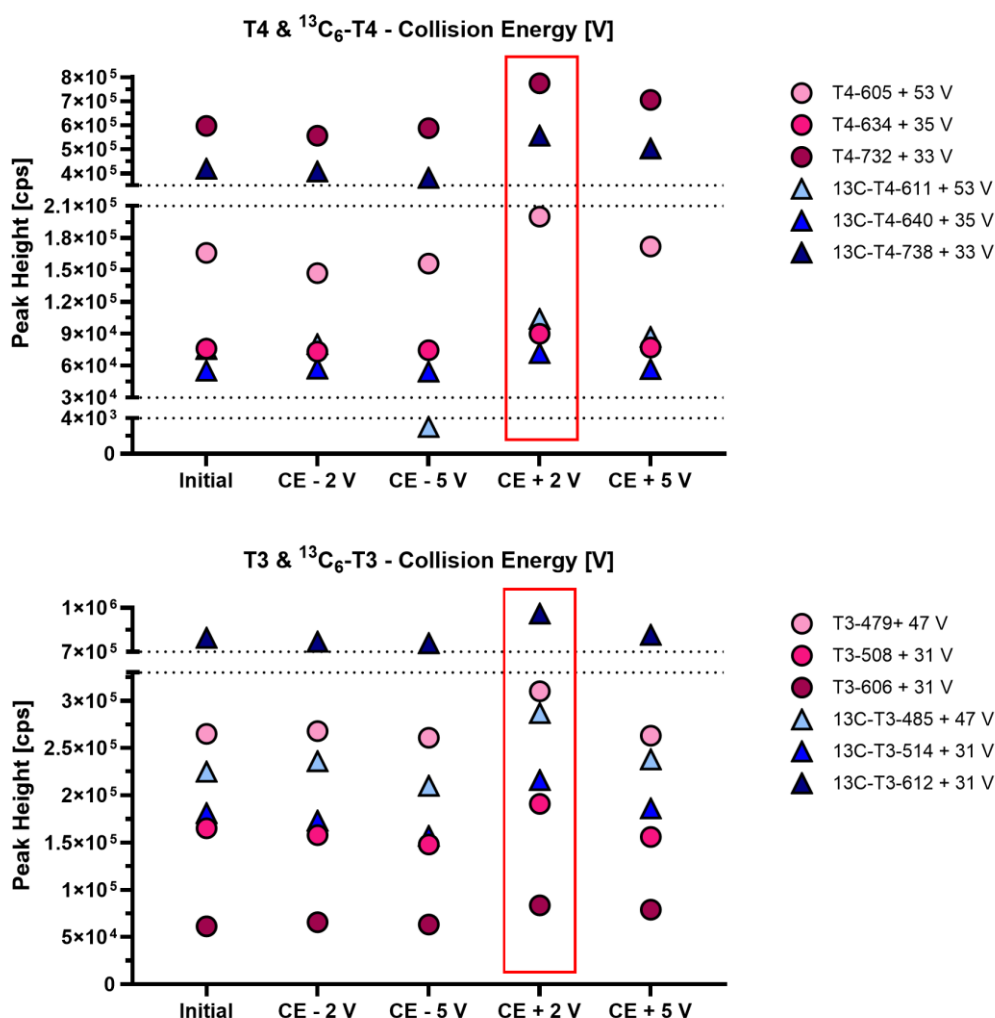


Figure 6. Peak height intensities of the last optimized MS parameter “Collision Energy” (CE) for analyte T4 and T3 and their isotope-labeled internal standards.

The top graph depicts results for T4 mass fragments and its isotope-labeled analog and below the results for T3 and $^{13}\text{C}_6\text{-T3}$ mass fragments. The y-axis shows peak height [cps] and, on the x-axis the examined CE as well as the initial settings are plotted. The initial CE values for every mass fragment is recorded next to its symbol in both legends. The examined energies are derived from there by adding and subtracting 2 V or 5 V as indicated on the x-axis. Dots refer to the mass fragments of the analyte and triangles to the mass fragments of the isotope-labeled analog. Final optimized CE value was selected by adding 2 V to the initial value which is marked by a red box in both graphs.

5.1 MS Optimization of Analyte Peak Intensities

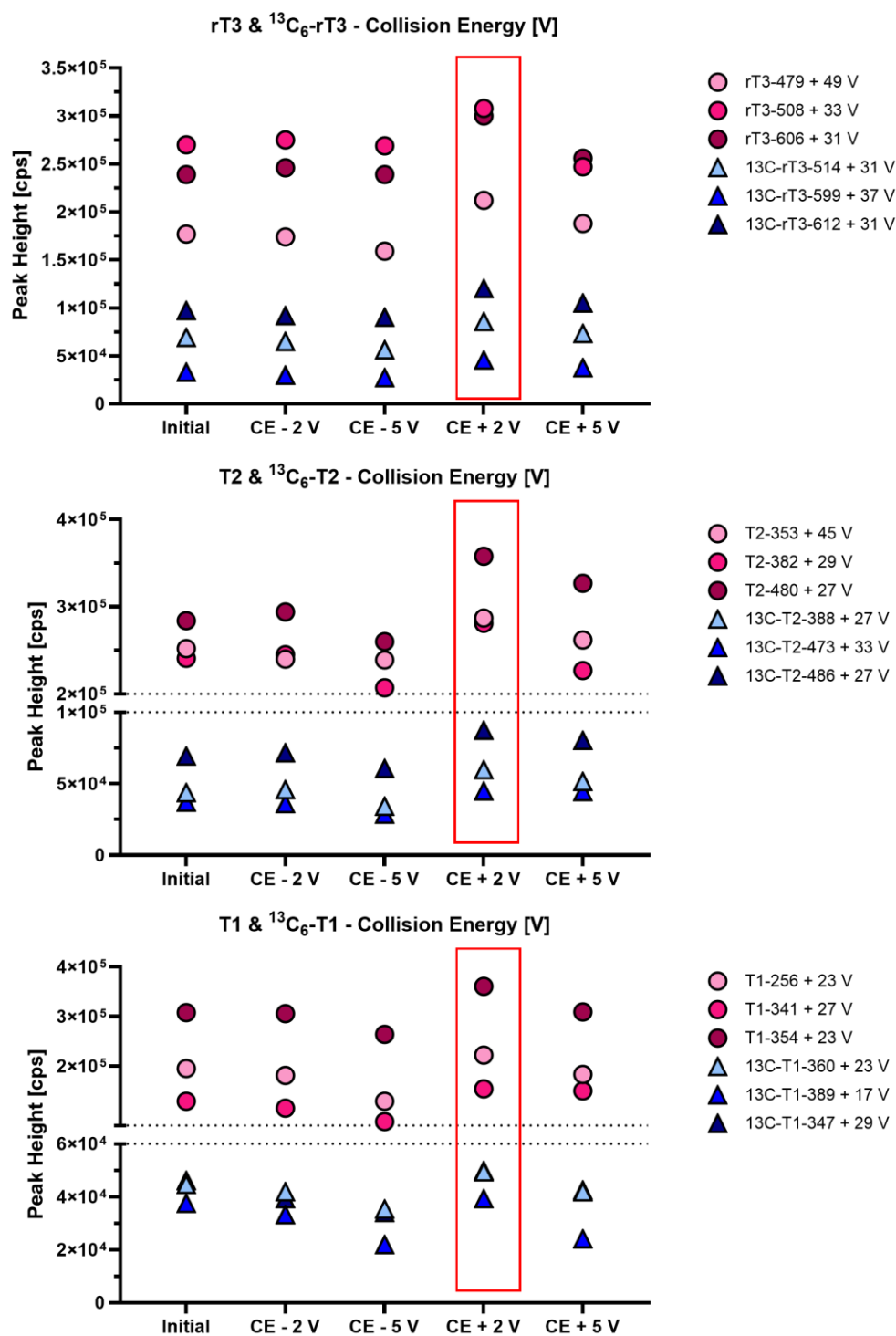


Figure 7. MS parameter “Collision Energy” (CE) optimization for rT3, T2, and T1 as well as their isotope-labeled internal standards.

The top graph depicts results for **rT3** mass fragments and its isotope-labeled analog and below the results for **T2** and **T1** and their respective isotope-labeled analog. The y-axis shows peak height [cps] and, on the x-axis the examined CE as well as the initial settings are plotted. The initial CE values for every mass fragment is recorded next to its symbol in both legends. The examined energies are derived from there by adding and subtracting 2 V or 5 V as indicated on the x-axis. Dots refer to the mass fragments of the analyte and triangles to the mass fragments of the isotope-labeled analog. Final optimized CE value was selected by adding 2 V to the initial value which is marked by a red box in both graphs.

5.2 Method Validation

5.2.1 Total THs in Rat Plasma

The results of the validation parameters are depicted in Table 17 and Table 18. As it can be seen all analytes fulfill the needed criteria which are additionally listed next to the parameter in the table below. Furthermore, the chosen calibration range including the lowest point, the LOD, and the LOQ are summarized. Notable, T4 incorporates higher concentrations compared to the remaining analytes which is because of the naturally higher concentration in plasma. Accordingly, LOD, LOQ, and the calibration range were adapted towards expected T4 concentration in plasma. Accuracies at LOQ and 10 x LOQ levels were determined within the needed 70-120 % range with an overall precision lower than 8 %. Specificity in analysis was established by not transcending 30 % of the analytes LOQ signal in neither the solvent nor in the PBS blank. Lastly, carry-over was reduced by including a blank sample after each plasma sample so that each analytes signal was not exceeding 30 % of its signal at LOQ level. In Table 18 the validation results for the additional analytes MIT, DIT, T4-Gluc, and T4-SO₄ are summarized which results all in all complete the given criteria, however T4-SO₄ did not fulfill criteria for the accuracy of the LOQ (below 70 %) and MIT as well as T4-Gluc resulted in accuracies at 10 x LOQ level slightly above 120 %. Overall, this method validation for plasma matrix was concluded as successful.

5.2 Method Validation

Table 17. Plasma method validation results for each category and analytes T1, T2, T3, rT3, and T4.
When no peak area was detected, the given cell is marked with “N/A”.

Section	Parameters	Criteria	T1	T2	rT3	T3	T4
	Quantifier	/	341	480	606	606	732
Standard solutions & Linearity	Calibration range [nM]	/	0.001-0.025	0.008-0.381	0.006-0.307	0.006-0.768	0.97-45.05
	R ²	≥ 0.99	0.9986	0.9979	0.9936	0.9985	0.9932
	Slope	/	0.23218	1.08280	1.04202	3.22434	0.75281
	Intercept	/	0.01890	0.01234	0.00259	0.04797	-0.000431
Accuracy & Precision	LOD [nM]	/	0.007	0.009	0.007	0.007	1.0
	LOQ [nM]	/	0.023	0.029	0.023	0.023	3.2
	Accuracy [%]	70-120 %	105.3	104.7	110.0	111.5	88.2
	RSD [%]	< 20 %	5.1	4.9	6.8	2.7	2.0
	10 x LOQ accuracy [%]	70-120 %	109.5	112.0	109.4	113.9	101.1
	10 x LOQ RSD [%]	< 20 %	3.7	7.4	5.9	3.3	2.7
Specificity	Solvent [%]	≤ 30 % of LOQ	N/A	8.1	7.3	12.4	1.4
	PBS Blank 1 [%]		N/A	N/A	28.6	28.0	1.2
	PBS Blank 2 [%]		N/A	N/A	N/A	6.9	4.1
Carry-Over	Blank after 1 st standard row [%]		N/A	N/A	14.0	22.2	0.3
	Blank after 2 nd standard row [%]		N/A	9.2	25.2	17.9	0.2

Table 18. Further validation results in plasma for analytes MIT, DIT, T4-Gluc, and T4-SO₄.
When no peak area was detected, the given cell is marked with “N/A”.

Section	Parameters	Criteria	MIT	DIT	T4-Gluc	T4-SO ₄
	Quantifier	/	135.0	387.9	732.0	732.0
Standard solutions & Linearity	Calibration range [ng/mL]	/	0.06-2.5	0.015-0.5	0.015-0.5	0.03-1
	R ²	≥ 0.99	0.9965	0.9934	0.99596	0.995
	Slope	/	0.02620	0.03960	0.00472	0.29775
	Intercept	/	0.02347	0.02841	-0.000598	0.05299
Accuracy & Precision	LOD [ng/mL]	/	0.06	0.015	0.015	0.03
	LOQ [ng/mL]	/	0.20	0.05	0.05	0.10
	Accuracy [%]	70-120 %	97.6	93.0	102.9	67.5
	RSD [%]	< 20 %	8.9	6.2	19.7	14.5
	10 x LOQ accuracy [%]	70-120 %	122.1	104.0	120.9	89.4
	10 x LOQ RSD [%]	< 20 %	11.1	9.3	5.4	3.9
Specificity	Solvent [%]	≤ 30 % of LOQ	N/A	N/A	N/A	9.0
	PBS Blank 1 [%]		N/A	N/A	N/A	29.0
	PBS Blank 2 [%]		N/A	N/A	N/A	N/A
Carry-Over	Blank after 1 st standard row [%]		N/A	14.8	N/A	26.2
	Blank after 2 nd standard row [%]		N/A	N/A	N/A	26.1

5.2 Method Validation

5.2.2 Total THs in Rat Brain

The same method validation procedure as described for plasma samples was done for the matrix rat brain except that analytes MIT, DIT, T4-Gluc, and T4-SO₄ were not included since they were not necessarily expected in this matrix. As recorded in Table 19, the analytes overall fulfill the criteria listed. LOQ and 10 x LOQ were predicted within the named range and with an overall RSD of lower than 10 % precisely ascertained. Due to the presence of all analytes in brain matrix, a matrix blank for rat brain was missing. Therefore, specificity was detected in a solvent blank which composed of the final resuspension solvent of the samples after evaporation. Like in the plasma method, a carry-over was detected and reduced by inserting blank samples between the brain samples. Two blank samples were included after standard concentrations above 2 ng/mL. Finally, method validation for TH analysis in rat brain matrix was achieved due to fulfilling the mentioned criteria in Table 19.

Table 19. Parameters, criteria and resulting values per analyte for brain method validation.

When no peak area was detected, the given cell is marked with "N/A".

Caption	Parameters	Criteria	T1	T2	rT3	T3	T4
	Quantifier	/	256	480	606	479	732
Standard solutions & Linearity	Calibration range [ng/mL]	/	0.01-0.3	0.005-0.5	0.005-0.3	0.05-2	0.05-4
	R²	≥ 0.99	0.99928	0.99373	0.99637	0.99936	0.99896
	Slope	/	0.61848	0.31903	0.25924	0.47977	0.56954
	Intercept	/	0.01777	0.02017	0.00205	-0.00721	-0.00233
	LOD [ng/mL]	/	0.006	0.006	0.006	0.045	0.045
Accuracy & Precision	LOQ [ng/mL]	/	0.02	0.02	0.02	0.15	0.15
	Accuracy [%]	70-120 %	106	110	90	99	99
	RSD [%]	< 20 %	9.1	5.7	5.0	2.4	1.8
	10 x LOQ accuracy [%]	70-120 %	99	116	98	92	104
	10 x LOQ RSD [%]	< 20 %	3.7	3.9	3.6	3.0	4.2
Specificity	Solvent [%]		N/A	N/A	N/A	N/A	4.9
Carry-Over	Blanks after 1st standard row [%]	≤ 30 % of LOQ	N/A	58.47	137.63	14.89	28.1
			N/A	N/A	34.29	3.09	8.76
	Blanks after 2nd standard row [%]		N/A	N/A	28.19	3.01	7.78
			N/A	N/A	11.86	1.7	6.38

5.2.3 Matrix Effect in Rat Plasma and Brain

Both used matrices contained naturally THs which is why it was not possible to verify for matrix effects for the analytes themselves. However, it is assumed that the added isotope-labeled internal standards follow the same pattern during sample preparation and analysis as the analytes. This is the reason why it is added to intercept deviations occurring during this procedure. Therefore isotope-labeled internal standard peak areas were compared between matrix and solvent samples which should not deviate more than ± 20 %. The results are depicted

5.3 Robustness of the Sample Preparation

in Table 20 demonstrating that there is a substantial matrix effect in particular in brain matrix. The seen matrix effect in plasma samples lied within the mentioned criteria. As previous mentioned the isotope-labeled standards were added to intercept any deviations including possible matrix effects which is why TH analysis in rat brain was proceeded.

Table 20. Determination of matrix effects in plasma and brain calculated per isotope-labeled standard.

Matrix	$^{13}\text{C}_6\text{-T1}$	$^{13}\text{C}_6\text{-T2}$	$^{13}\text{C}_6\text{-rT3}$	$^{13}\text{C}_6\text{-T3}$	$^{13}\text{C}_6\text{-T4}$
Plasma	- 20.0 %	- 11.6 %	19.0 %	- 18.5 %	- 20.0 %
Brain	- 72.0 %	- 77.0 %	- 79.0 %	- 77.0 %	- 82.0 %

5.3 Robustness of the Sample Preparation

Pooled plasma and brain samples were generated to normalize each analyte's concentration independent of sex to get a better view of fluctuation possibly caused by sample preparation. For each matrix pool from one study, matrix control samples from ten females and ten males were combined to generate a pool from which technical replicates were included in each measurement. Consistency in sample preparation was verified by RSD for which a low value reflected low variability between the technical replicates. In Table 21 all RSDs of all analytes and matrix pools are listed and sorted per sampling day (d) and age. A low RSD was defined as below 20 % orientated from RSD criteria regarding precision in method validation. Especially analytes T3 and T4 were in every group below 20 % and therefore it is assumed that sample preparation is robust and did not majorly influence variation within a sample group. Analytes rT3 and T2 were above 20 % in three of seven groups for which it needs to be mentioned that T2 concentration in plasma were mostly below its LOQ value. A better overview of fluctuation between different pools generated in different years which were measured alongside individual sample analysis during this project are presented for the technical replicates of each adult plasma pool per analyte in Figure 8. Symbols indicate the different analytes for which dots depict T4, squares T3, triangles rT3, and stars T2. Overall, 99 technical replicates were detected whereas T2 concentration could be detected in 93 out of 99 because of the previous mentioned reason. The technical replicates were divided per year of study pool which was also visually separated by red solid lines. The corresponding year of every study from which pools were generated are written within each section. Usually in sections including more than five technical replicates more than one pool from the given study were analyzed. T4 yielded an RSD of 14.2 % in adult plasma pools which is reflected in Figure 8 by the dots (T4). A decline to the last section is visible for analytes T3, rT3, and T2, however prior to the last section they obtained steady values. This discrepancy affected the corresponding RSD calculation which resulted in higher values for the remaining analytes except for analyte T4 which were as follows 21.1 % T3, 32.7 % rT3, and 38.4 % T2. The last section of technical

5.3 Robustness of the Sample Preparation

replicates was measured within the beginning of method usage (beginning of project) and thus might be associated to sample handling being less proficient even though this seemed to only affect T3, rT3, and T2 since it was not reflected in T4 values.

Taking everything into account, it was concluded that sample preparation was robust and consistent for all analytes whereas rT3 and T2 were more prone to higher variation than T4 and T3.

Table 21. RSD in percentage for each analyte and matrix pool from all studied examined and sorted per sampling day [d].

T1 concentration was not detected in none of the investigated samples and the cells are marked with a slash symbol.

RSD [%]/Analyte	T1	T2	rT3	T3	T4
Brain (adult after 28d, n=25)	/	28.3 (n=14)	31.3 (n=17)	13.9	18.5
Brain (adult after 7d, n=13)	/	22.6 (n=10)	15.9	16.5	16.9
Plasma (PND21, n=19)	/	18.9	23.3	7.8	10.1
Plasma (Adult after 28d, n=99)	/	38.4 (n=93)	32.7	21.1	14.2
Plasma (Adult after 7d, n=9)	/	12.7	15.4	3.9	8.5
Plasma (F2, n=5)	/	9.2	6.3	5.1	8.1
Plasma (F3, n=4)	/	9.9	9.03	1.6	3.1

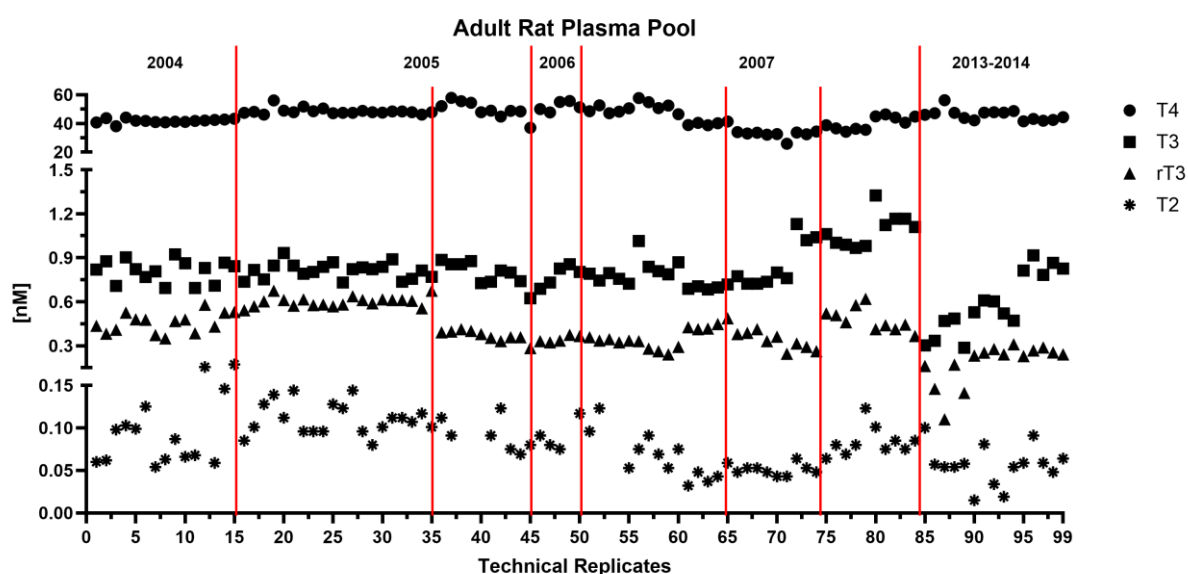


Figure 8. TH concentrations of technical replicates from various study rat plasma pools sorted per year. Analytes are depicted as different symbols such as dots refer to T4, squares to T3, triangle to rT3, and stars to T2 which order also indicates the decrease in concentration. Technical replicates are divided by study pool and year visible by the red solid lines. Furthermore, the manufacturing year of each study pool is indicated above its section. In total, a number of 99 technical replicates were measured in which only T2 was not detected in all of them.

5.4 Method Development and Detection of Free THs in Rat Plasma

5.4.1 Comparison of Two Method Approaches for Free TH Analysis

The free TH fraction in blood is less than 1 % of the total concentration and actively enters tissue cells to mediate gene transcription. Method development to analyze the free fraction in plasma was established to estimate whether changes in TH concentrations caused by a substance might be earlier detectable in the free fraction. While examining the potential of both method approaches (see descriptions 4.1.5.1 & 4.1.5.2) mostly spiked PBS with physiologically TH concentrations was utilized because of the high volume needed, the limited availability of rat plasma samples and the lack of analyte free matrix namely plasma without TH as described in section 4.1.5. In these following subsections 5.4.1.1, 5.4.1.2, and 5.4.1.3 the isotope-labelled internal standard mix (IS-mix) was added every time after the separation method meaning prior to evaporation of the given sample. Thus, potential analyte loss during the separation procedure was not intercepted by the internal standard.

5.4.1.1 Ultrafiltration (UF) Method

During development of the UF method three parameters were optimized: centrifugation force, sample volume and washing steps of filter. Prior to starting with the optimization an initial experiment was tested with parameters obtained from the Centrifree® Ultrafiltration Device User Guide shown in Figure 9A. The User Guide recommended using a fixed-angle rotor centrifuge rotor however due to non-availability, a swinging-bucket centrifuge rotor (Allegra X-15R, Beckman Coulter GmbH, 47807 Krefeld, Germany) was used. This approach did not include an evaporation of the ultrafiltrate whereas a filtrate of around 120 µL was taken and a total volume of 200 µL was prior loaded onto the filter. Based on these results evaporation after filtration was reintroduced and the centrifugation force was first assessed while simultaneously testing different loading volumes. The samples were either centrifuged with 1,000, 1,500, or 2,000 x g in a loading volume of 200 µL or 300 µL. As seen in Figure 9 B & C the calculated recovery was the highest at 1,500 x g with which further optimization was continued. In a last step, the filter was either coated with a solution to prevent binding to polymer surfaces (Biofloat™ Clean Coating Solution, faCelliate, Chemovator GmbH, 68169 Mannheim, Germany), pre-conditioned with filtrating Millipore water, the eluate cup was rinsed afterwards with MeOH, or a combination of the last two steps to increase recovery of the analytes. No significant improvement was achieved for none of the analyte's recoveries as seen in Figure 9D, however since T1 was detected when pre-washing the filter membrane with Millipore water subsequent experiments included this step.

5.4 Method Development and Detection of Free THs in Rat Plasma

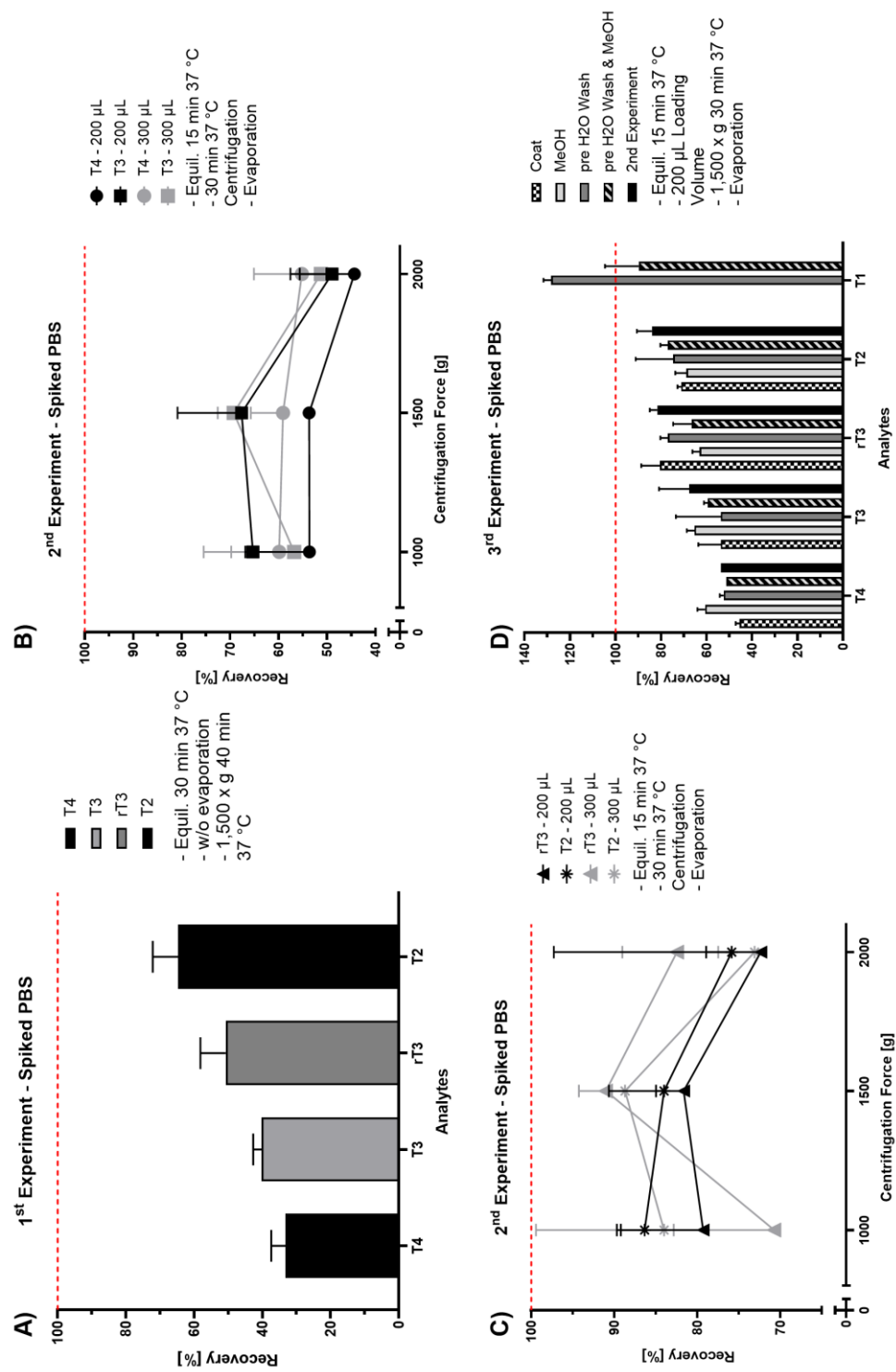


Figure 9. Overview of the results from the method development for fTHs using UF.
A) Recovery results from first experiment oriented on Device User Guide. B) Optimization of centrifugation force for T3 and T4 using a loading volume of 200 μ L or 300 μ L. The recovery is determined of the spiked amount found in the given sample. C) Same experiment conditions as for B) just that results for rT3 and T2 are shown. D) Different conditions focusing on the filter unit itself were investigated and calculated recoveries are summed up in this graph.

5.4 Method Development and Detection of Free THs in Rat Plasma

5.4.1.2 Rapid Equilibrium Dialysis (RED) Method

The same development approach principle was applied to the RED method. First an initial experiment was conducted orientated on the available RED User Manual. Like the UF approach, PBS was spiked in physiological TH concentrations for RED to determine their recoveries by calculating measured TH concentrations. Parameters optimized for RED were incubation time and mixing rate. In Figure 10A all calculated recoveries were below 100 % and the lowest was obtained for T4 (around 40 %). In the first experiment the removed sample volume from the chamber was not evaporated which step was reintroduced afterwards. The testing of different incubation times and mixing rate gained a small increase in recoveries as seen in Figure 10B & C.

5.4 Method Development and Detection of Free THs in Rat Plasma

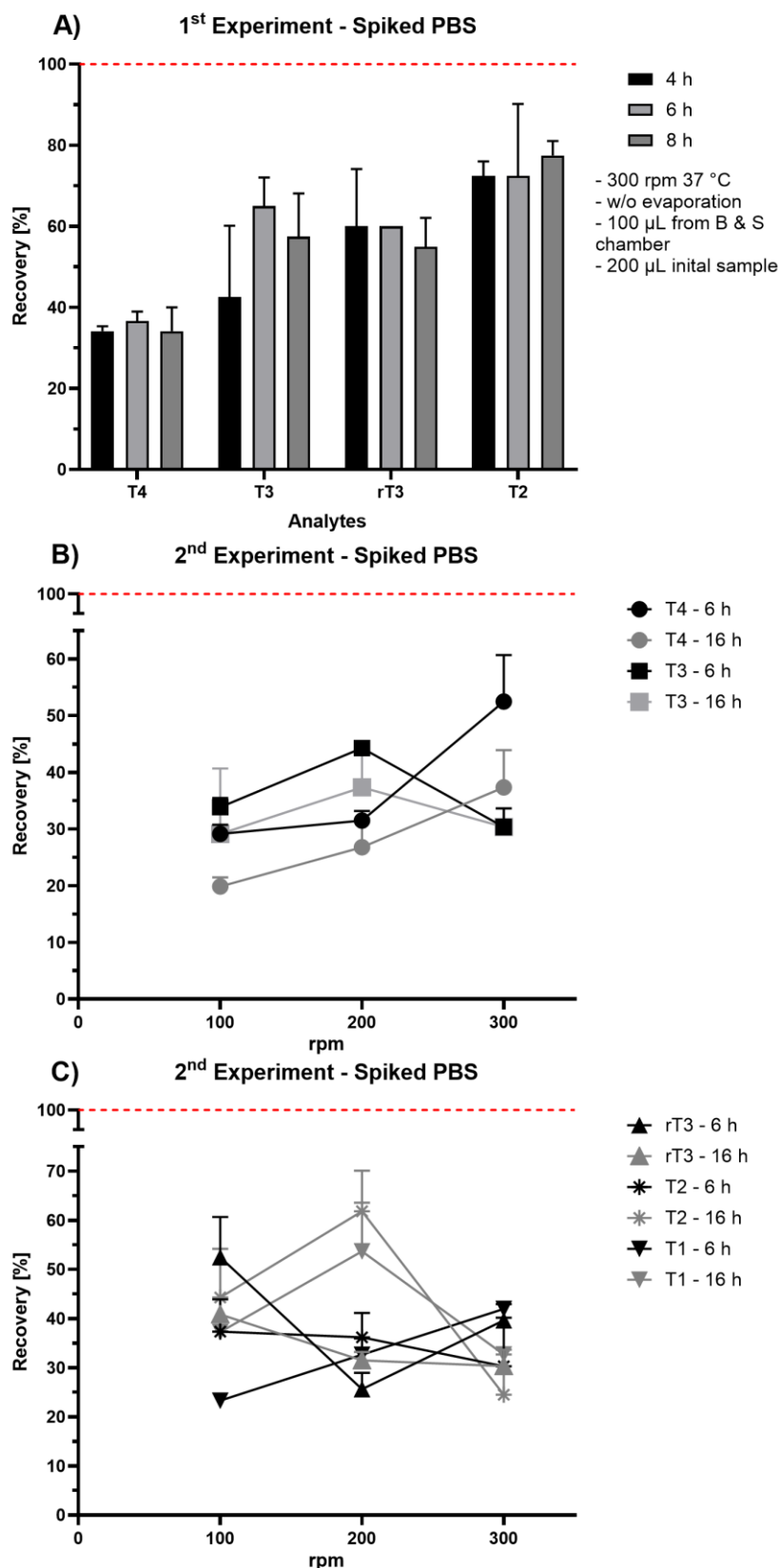


Figure 10. Graphical illustration of recovery results applying RED approach.

A) Initial experiment to examine handling of method and estimate TH recoveries. B) T3 and T4 recovery calculations after incubating for 6 h or 16 h as well as at different mixing rates. C) The graph shows recovery results for remaining analytes rT3, T2, and T1 under same conditions as described under B).

5.4 Method Development and Detection of Free THs in Rat Plasma

5.4.1.3 Comparison of Methods Regarding Free TH Detection in Rat Plasma Pool

After optimizing both method approaches, a first trial analyzing rat plasma pool was implemented. Rat plasma pool volumes of 200 μL and 300 μL were examined with UF and RED approach. The RED approach was additionally incubated for either 6 h or 16 h with both volumes. The final sampling from the chambers were not only taken out of the “Buffer chamber” obtaining fTH fraction, but also out of the “sample chamber” which contained the initial added plasma sample meaning the protein-bound fraction. The final UF approach involved pre-washing of the filter membrane with Millipore water, and was followed by centrifugation of the plasma sample at 1,500 x g for 30 min at 37 °C. All samples were evaporated to dryness and resuspended in 140 μL aqueous MeOH before analysis. Free fraction was detected for analytes T3 and T4 in both method approaches while rT3 and T2 were also quantified measuring the protein-bound amount using RED depicted in Figure 11. The detected free fraction was $\leq 2\%$ of the protein-bound TH fraction for T3 and T4 whereas for the remaining analytes no free fraction was determined.

5.4 Method Development and Detection of Free THs in Rat Plasma

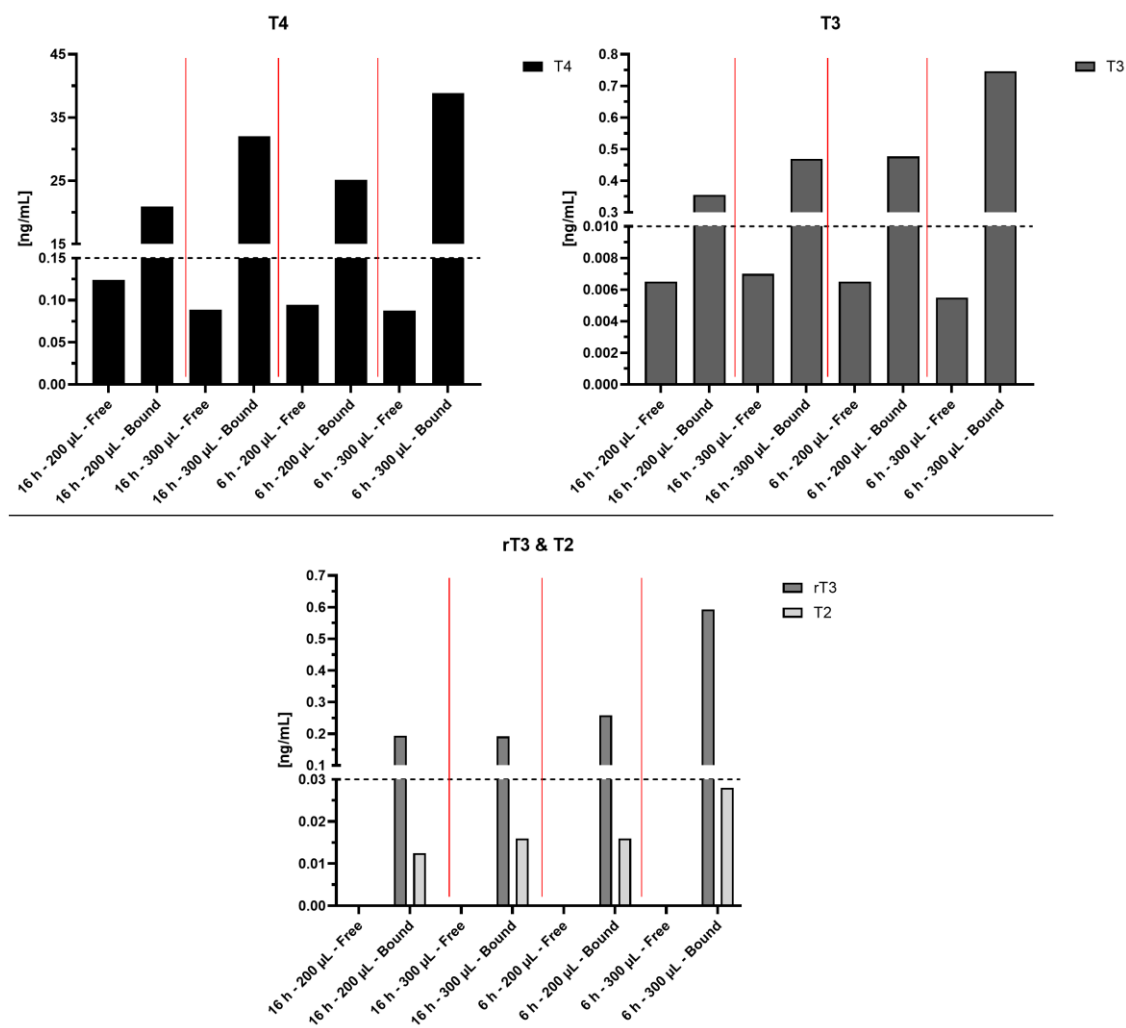


Figure 11. Analysis of free and bound fraction of THs in rat plasma pool using RED.

In each bar graph the free and bound fractions (duplicates per sample volume and incubation time) are plotted on the x-axis against the corresponding concentration [ng/mL] on the y-axis. On the top left results for **T4** are depicted, on the top right **T3**, and below results for analytes **rT3** and **T2**. The red solid lines further visually partition the samples paired by volume and incubation time examined. No free **rT3** and **T2** was detected.

5.4 Method Development and Detection of Free THs in Rat Plasma

In Figure 12 detected fTH concentrations are presented after testing different plasma volumes and testing under different experimental conditions using either UF or RED. The methods are visually divided by a red vertical line and a black vertical line separated T4 from T3 results. It was striking that final concentrations detected using UF were around three times lower than the concentrations detected using RED. The method approach RED was selected for continuation of method development.

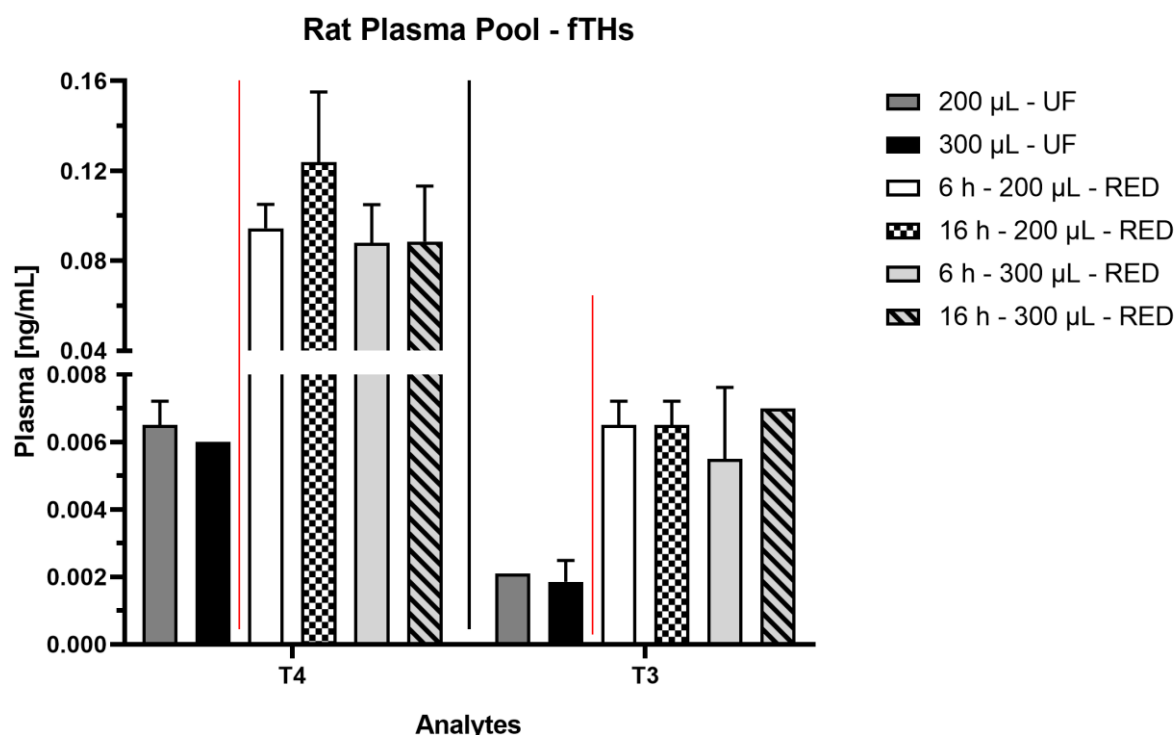


Figure 12. Detection of fTHs in rat plasma pool using UF and RED approach.

The concentration [ng/mL] is plotted against the analytes T4 and T3 on the x-axis. The different colored bars reflect different parameters such as method used, different sample volume, and/or incubation time. The results obtained using UF are separated from the results using RED by a red vertical solid line whereas analytes are divided by a black line. The patterned bars refer to an incubation time of 16 h using RED and the white and light grey colored bars of 6 h using either 200 or 300 µL plasma.

5.4.2 Addition Isotope-Labelled Internal Standard – Yes or No?

An IS-mix was so far always included from the beginning of sample preparation to adjust for analyte loss during the entire procedure. For method development regarding fTHs, an IS-mix was included after RED or UF due to concerns of competition with the analytes over binding sites on the plasma TH-binding proteins in plasma¹³⁶. With this handling the IS-mix corrected for any loss after RED or UF but did not cover loss during the use of the RED or UF approach. To enable correction of analyte loss during the entire procedure, RED experiments were conducted with physiologically spiked PBS including either all isotope-labelled internal standards (IS), only ¹³C₆-T1 or solely ¹³C₆-rT3. Single incubation with either ¹³C₆-T1 or ¹³C₆-rT3 were chosen because of the structural similarity to the analytes T4 and T3 as well as the

5.4 Method Development and Detection of Free THs in Rat Plasma

hypothesis that the impact on protein-binding-site competition is less than with their corresponding isotope-labelled analog. Besides single incubation with $^{13}\text{C}_6\text{-T1}$, duplicates were tested from which chambers were pre-rinsed with a $^{13}\text{C}_6\text{-rT3}$ solution to cover possible polymer binding sites and thus increase analyte recoveries. The recovery of duplicates was determined and compared between the mentioned conditions. Since the investigated samples were spiked in PBS, a sample was taken out of both RED chambers so that in total an amount of four values was gained. When plasma samples were analyzed, sampling was done only out of the buffer chamber. Figure 13 demonstrates the obtained recoveries for each analyte under each listed condition. The data were evaluated with the analytes corresponding IS analog for the samples including the entire IS-mix (light and squared bar in Figure 13). All analytes in the duplicates including solely $^{13}\text{C}_6\text{-T1}$, and the duplicates pre-rinsed with $^{13}\text{C}_6\text{-rT3}$ were evaluated using $^{13}\text{C}_6\text{-T1}$ (striped and black bars). The analytes in the last duplicates including only $^{13}\text{C}_6\text{-rT3}$ identified as dark grey bars were all evaluated using $^{13}\text{C}_6\text{-rT3}$ accordingly. Overall, all data consisted of four values since each chamber was analyzed except for the samples in which the IS-mix was added after dialysis. Since the IS-mix was added after dialysis, it could not adjust for analyte loss during dialysis, therefore the values yielded from sample and buffer chamber were added up. The high rT3 concentration in the samples pre-rinsed with it IS analog was likely caused by contamination of rT3 in the high-concentrated $^{13}\text{C}_6\text{-rT3}$ solution and was therefore not considered further. The pre-rinse using $^{13}\text{C}_6\text{-rT3}$ solution did not increase recovery for the analytes investigated, therefore this condition was not pursued further. The recoveries obtained when adding the IS-mix after dialysis (light bar), and during dialysis solely $^{13}\text{C}_6\text{-rT3}$ (dark grey bar) or solely $^{13}\text{C}_6\text{-T1}$ (black bar) yielded similar result for analyte T4. The last two conditions improved recoveries for analytes T3, rT3, T2, and T1 whereas no T2 was detected with solely $^{13}\text{C}_6\text{-rT3}$, and T1 was overpredicted when incubating with IS. This overprediction was additionally detected for all analytes except T2 when the entire IS-mix was added during dialysis and thus accordingly evaluated with the analytes IS analog (squared bar). Since the best results were attained using either $^{13}\text{C}_6\text{-rT3}$ or $^{13}\text{C}_6\text{-T1}$, it was concluded that a trial experiment in rat plasma using either one of the mentioned IS should be carried out.

5.4 Method Development and Detection of Free THs in Rat Plasma

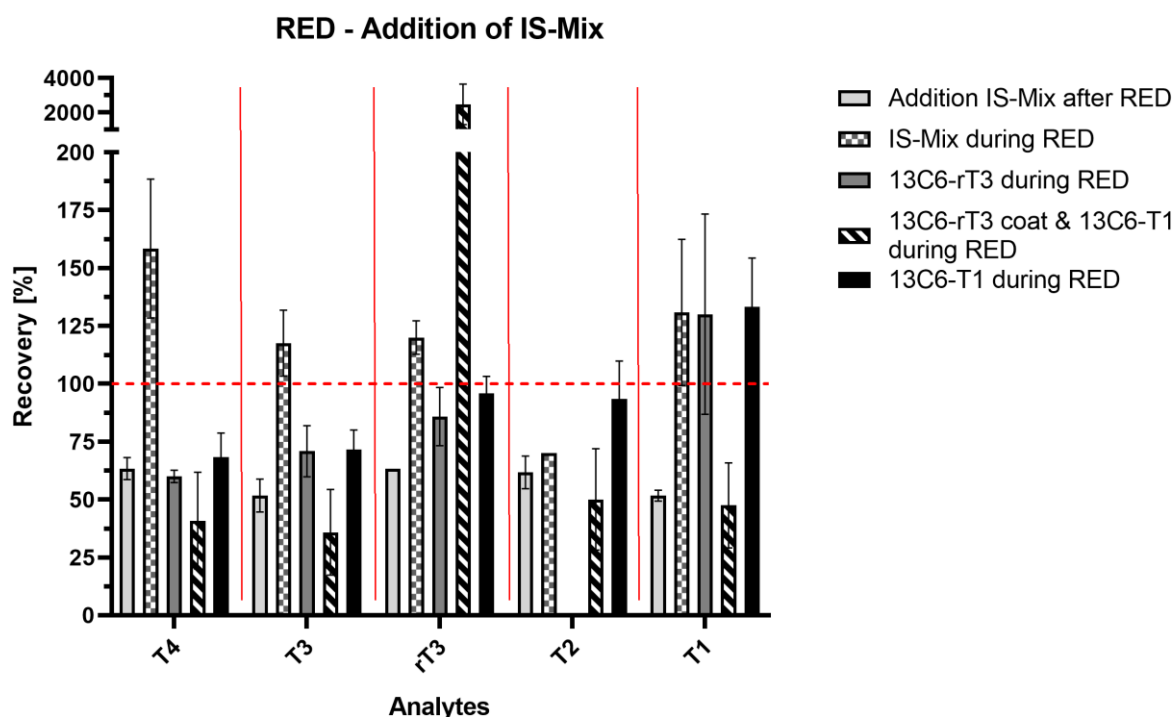


Figure 13. Recovery results gained after different additions of IS during RED.

The y-axis shows yielded recovery in percentage and on the x-axis each analyte investigated. The different patterned bars refer to different conditions regarding the handling of isotope-labelled internal standard. The lightest bar portrays the initial condition meaning addition of the IS-mix after dialysis whereas in the squared bar the IS-mix was included in dialysis. The following three bars only contained one IS therefore the data were evaluated with the mentioned IS. When the IS-mix was used containing all IS, the data were evaluated with the corresponding isotope analog.

In addition to the calculated recoveries, all analytes and IS area counts are portrayed in Figure 14. The previously mentioned potential analyte loss was confirmed when comparing IS area from experiment conditions “addition IS-Mix after RED” to the remaining test conditions including either IS-mix or one IS during RED. In Figure 14A) calculated IS area loss ranged from 64 % to 81 % due to the assumption that IS and analytes acted in a similar pattern, it might be concluded that analyte area loss during RED was deemed to be in a similar range. The analyte areas themselves seemed not to be affected in the different experiment conditions (see Figure 14B)). In the bottom graph C, the above pointed out potential contamination of rT3 in the high-concentrated ¹³C₆-rT3 solution was visible by the high rT3 area counts in the pre-rinsed samples compared to the other analyte area counts which appeared to be unaffected by the high ¹³C₆-rT3 concentration used to pre-rinse.

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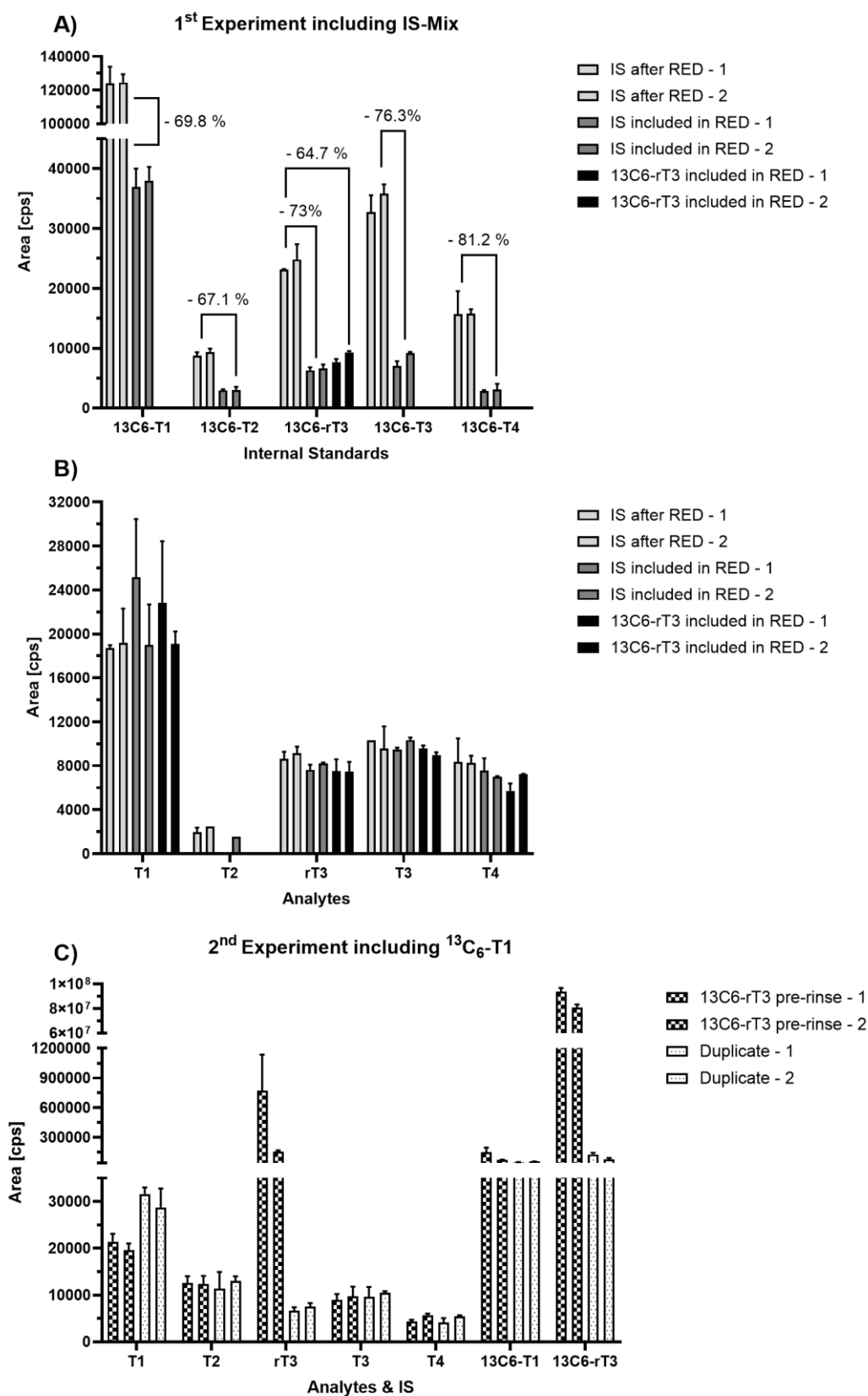


Figure 14. Overview of peak areas gained from the first and second RED experiment including the IS-mix or one single IS.

All bars consisted of duplicates (n=2) meaning samples were taken out of the buffer and sample chamber as well as every experiment condition was tested in duplicates. A) and B) includes the area counts [cps] from the first

5.4 Method Development and Detection of Free THs in Rat Plasma

experiment which involved adding the IS-mix after RED (light grey), adding the IS-mix during RED (dark grey) or adding solely $^{13}\text{C}_6\text{-rT3}$ during RED (black). A) The top graph depicts the area counts for every IS whereas in the last condition only $^{13}\text{C}_6\text{-rT3}$ was obtained. In addition, the area loss in percentage is noted between adding the IS-mix after or during RED for each condition. B) The graph in the middle shows the corresponding analyte areas from the first experiment. C) The second experiment involving a pre-rinse with $^{13}\text{C}_6\text{-rT3}$ (black squared) of the chambers and only adding $^{13}\text{C}_6\text{-T1}$ for evaluation during RED (white dotted) is pictured on the bottom showing the area counts for all analytes and the two mentioned IS.

5.4.3 Determination of Free TH Concentration in Individual Control Animals and Pooled Samples

The next step included the determination of variability regarding technicality but also between the animals. In both experiments the internal standard $^{13}\text{C}_6\text{-T1}$ was included during RED to adjust for the previous detected analyte loss. Technical variability was estimated by calculating the RSD from four technical replicates of a rat plasma pool. Due to the higher volume needed for one replicate (200 μL), three plasma pool vials were mixed and from this mixture technical replicates were taken for RED procedure. The precision was estimated to be around 11 % for free T4 (fT4) and 13 % for free T3 (fT3) which reflected overall low technical influence. Therefore, it was proceeded to analyze plasma of male and female control animals at different sampling days (d) referred to as 7 d, 14 d, and 28 d. Each sampling day consisted of five biological replicates of males and females respectively except for males on day 28 which consisted of four replicates. In Table 22 RSD data for plasma pool replicates and individual control animals are summarized. Overall, there was no exceptionally large variability within all data sets. Female rats on day 7 and day 28 displayed the largest variability for T4 with 35 % and 40 % respectively which was not reflected in the RSD of T3. The remaining sampling days illustrated similar RSD between both analytes.

Table 22. List of calculated RSD values shown as percentages in plasma pool and control samples.

RSD [%]	fT4	fT3
Pool (n=4)	10.9	13.0
Males (7d and 14d, n=5; 28d, n=4)	19.4; 24.5; 14.5	11.5; 20.8; 14.3
Females (7d; 14d; 28d, n=5)	35.2; 18.6; 40.1	14.1; 13.1; 7.5

The mentioned IS area loss in the previous section was investigated in actual plasma pool samples too. In two experiments, four technical replicates of a pooled rat plasma pool were equilibrated using RED whereas in one experiment the IS was simultaneously incubated, and in the second experiment the IS was added after RED. As depicted in Figure 15 the area loss for IS (bottom) and the concentration difference for fT4 and fT3 (top) was plotted. Overall, both calculated reductions of either concentration or peak area laid within a similar range of around 87-94 % whereas the peak area of IS added after RED was similar to the peak areas of the pipetted standard sample symbolized as grey dots and triangles for experiment one and two.

5.4 Method Development and Detection of Free THs in Rat Plasma

Since the standard samples were pipetted directly without undergoing RED the IS area count was similar to the IS area in the plasma pool samples when the IS was added after RED.

In conclusion, this means that around 90 % of the initial amount was lost during RED and that it can be assumed that this was accurate for the analytes in a similar manner. Therefore, further fTH analysis was proceeded using $^{13}\text{C}_6\text{-T1}$ included in RED.

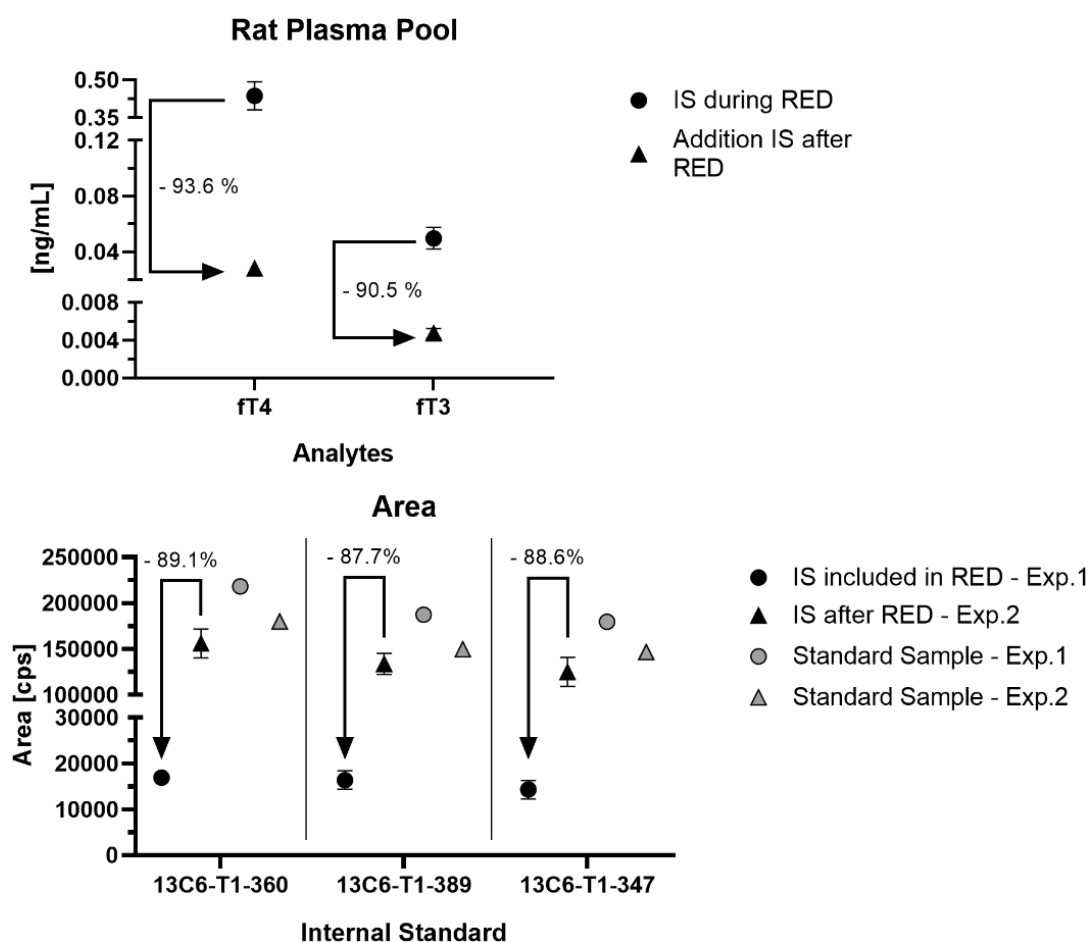


Figure 15. Graphical comparison of analyte and IS loss depending on timepoint of IS addition.

On top the calculated concentrations of free T4 and T3 are shown when adding the IS during (black dots) or after RED (black triangles). Correspondingly the graph below plots the peak area counts of all transitions of IS $^{13}\text{C}_6\text{-T1}$ added during or after RED. The graph below additionally shows the area count in standard samples for each experiment (grey dot and triangle). The symbols dot and triangle differentiate between experiment 1 and 2. In both graphs the decline in concentration and the area loss are presented in percentage.

5.4.4 Free TH Alterations after Exposure to PTU and Aroclor 1254

Samples from two studies administering either PTU or Aroclor 1254 previously analyzed regarding their total TH concentrations in rat plasma were additionally investigated for their free TH concentrations. These substances were selected as positive controls due to their strong effect towards total TH concentrations as well as they represented two different TH-related MOAs. According to the sample preparation protocol, 200 μL plasma should have been used

5.4 Method Development and Detection of Free THs in Rat Plasma

for RED, however most of these plasma samples only yielded 180 μL which was therefore used for analysis. The 180 μL plasma volume was first pipetted into a 2 mL Eppendorf Tube, 20 μL of the internal standard $^{13}\text{C}_6\text{-T1}$ solution was added and afterwards pipetted into the sample chamber of the RED plate. Into the buffer chamber next to the sample chamber, 400 μL PBS buffer was added which was prior reheated to around 37 $^{\circ}\text{C}$. The samples were incubated for 6 h at 300 rpm at 37 $^{\circ}\text{C}$ after which around 180 μL was removed out of the buffer chamber and analyzed after evaporation and resuspension. Due to generally limited volume left in the stored plasma samples only five controls per sex and the high dose (HD) (n=5) were examined. HD plasma samples from male rats exposed to PTU were not sufficient for analysis and thus only plasma from female rats were analyzed. Additionally, from each study five technical replicates of plasma pool samples were prepared. In Figure 16 the results from both studies as well as from a study from which only control animals were analyzed are presented. Red solid lines are dividing the data sets between the studies which are also written above the box plots as well as whether the data set was from females or males. The blue and red color of the dots refer to either male or female animals from which the control groups have a brighter color than HD groups. Plasma sampling was conducted on three different days (7, 14, and 28) which are listed on the x-axis. The first data set in each graph involves results from male and female controls on the three sampling days. The fT4 and fT3 concentrations over the sampling days were coherent and similar to the controls analyzed in the other studies. The first substance analyzed was PTU for which the control group and HD group of females were analyzed regarding their fT4 and fT3 concentrations. HD of PTU strongly decreased fT4 and fT3 concentrations compared to the control group. Half of the measured concentrations of both analytes in the HD samples were below LOQ which further emphasized the strong decrease in concentration which caused a decline of around 94-97 % for fT4 and around 58-81 % for fT3. A similar decrease in fT4 concentration ranging from 50-80 % compared to the given control was further detected for rats exposed to Aroclor 1254 on all sampling days. On contrary, fT3 was not majorly affected by Aroclor 1254 administration except for day 14 in females and day 7 in males showing a significant alteration. Interestingly, fT3 concentration in males tended to increase whereas in females the opposite was occurring. The last-mentioned observation was in contrast to the decline of total T3 concentration in females and males as described in section 5.7. However, the general observations with an overall decrease after PTU or Aroclor 1254 exposure were reflected in total as well as free TH analysis.

5.4 Method Development and Detection of Free THs in Rat Plasma

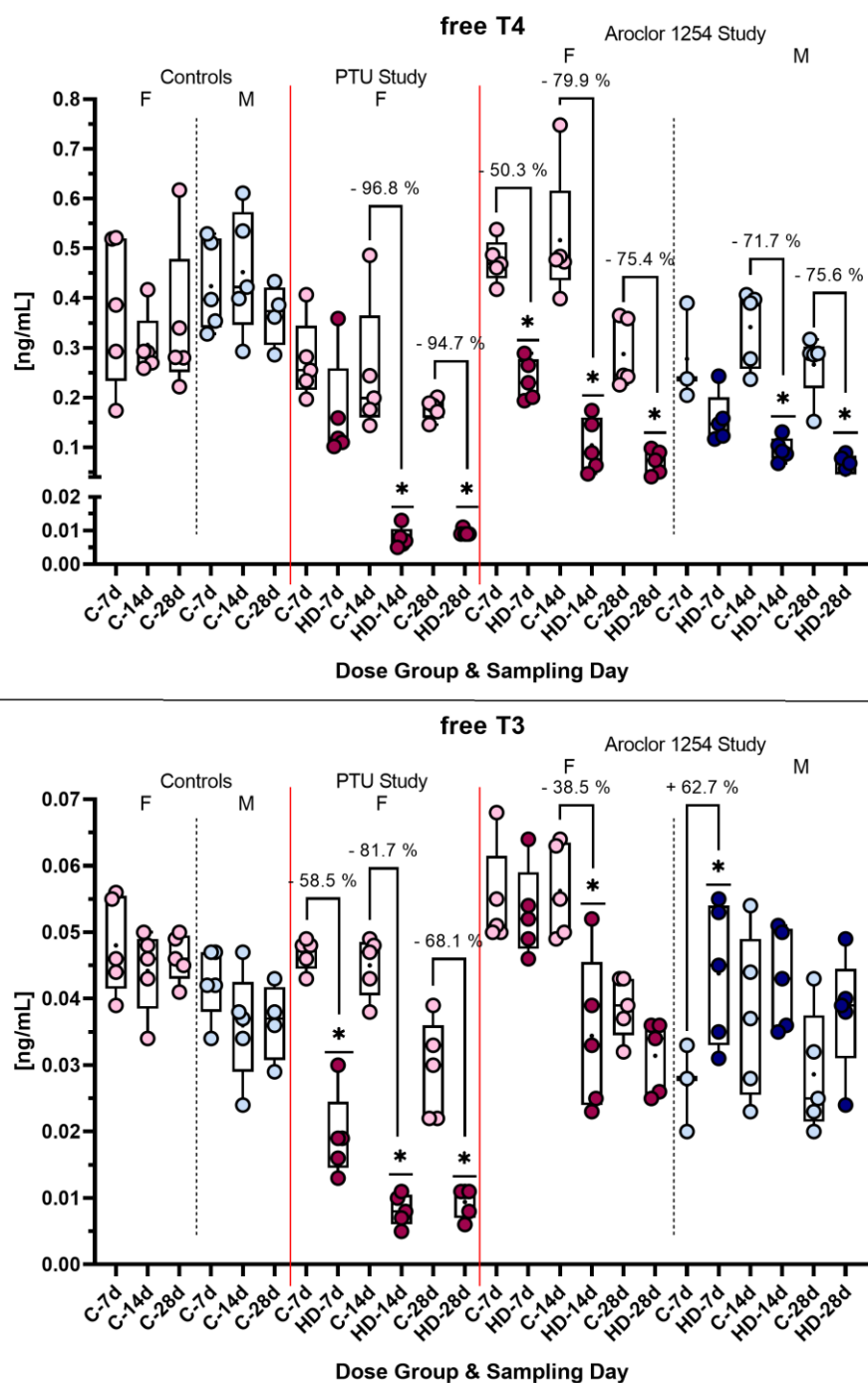


Figure 16. Free T4 and T3 analysis in plasma of control and PTU-, Aroclor 1254-exposed rats.

The top box plot graph encompasses the results for **free T4** and the graph below for **free T3**. Both graphs include results from three different studies which are visually separated by a red solid line. The first study entitled "Controls" includes analysis of only control animals at three sampling days. The other two studies are named after their respective exposure substance. Above the box plots the sex is indicated as well as this is recognizable by the blue and red colored dots for either males or females. Control groups additionally are marked with a brighter blue or red color compared to the HD data sets. Statistical significance was calculated from the respective control group of the same sampling day and is indicated by the asterisk defined as a p value at $* \leq 0.05$. For these significant alterations the respective percentage of concentration decline or increase compared to the control group is mentioned between the respective groups.

5.5 TH Detection in Different Rat Strains

For a better understanding of TH variation between different rat strains, rat plasma from four different strains were analyzed: Wistar Han (CrI:WI(Han)), Sprague Dawley (CrI:CD(Sprague Dawley)), F-344 (F-344/CrI) and Wistar Han RCC (RccHan:WIST). Differences between the strains are once that F-344 rats are an inbred rat strain compared to the others being outbred strains. Wistar Han RCC compared to Sprague Dawley and to F-344 rats have lower tumor incidence and Wistar rats have a detailed compilation of historic control data for various parameters¹³⁷. The Wistar Han RCC rats were originally derived from the Wistar colony kept at “Zentralinstitut für Versuchstierzucht” in Hannover in Germany from which Harlan Sprague-Dawley Inc. in Indianapolis in the USA in 1993 received a colony of Wistar rats namely the HsdHan:WIST¹³⁸. In 2004 this company acquired RCC Ltd. in Itingen in Switzerland to which the breeding stock was transferred as RccHan:WIST strain^{138, 139}.

Male and female plasma samples (n = 5) were taken from internal storage (inert gas atmosphere at -80 °C) and analyzed at three sampling days: 7, 14, and 28. In parallel to analysis of the different control samples, technical replicates of two different rat strain plasma pools were included to examine for variation introduced by sampling preparation. The composition of the plasma pools consisted of mixing female and male plasma as described in section 5.3. In Figure 17 the calculated analyte concentrations are plotted whereas dots represent T4, squares T3, triangles rT3, and stars T2. The examined plasma pools were from F-344 (left) and Wistar Han (right) rats which were visually separated by a red solid line within the graph. Overall, combined RSD of the analyzed technical replicates for T4 and T3 fell below 20 % whereas for rT3 and T2 it was above 20 %. Calculation of RSD per rat strain pool for rT3 yielded around 5 % for both rat strains individually and for T2 9.9 % for F-344 and 21.2 % for Wistar Han technical replicates.

5.5 TH Detection in Different Rat Strains

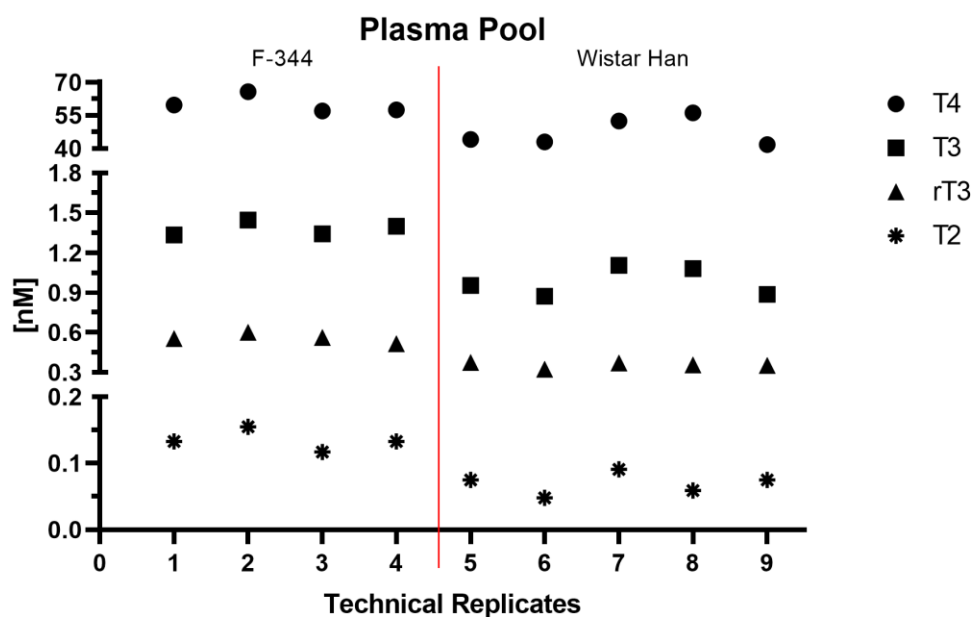


Figure 17. TH analysis in two different rat strain plasma pools.

To examine technical reproducibility, technical replicates were prepared from two rat plasma pools. These two plasma pools are visually divided by a red solid line whereas results from technical replicates from F-344 plasma pool are on the left and on the right from Wistar Han plasma pool. Analytes are identifiable by symbols of which dots represent T4, squares T3, triangles rT3, and stars T2. On the x-axis each technical replicate is listed and on the y-axis, the respective concentration [nM].

The respective results of each individual animal for T4 and T3 are demonstrated in Figure 18 and for the remaining analytes in Figure 19. The data sets are divided by rat strain which is indicated by the black dotted lines as well as within one data set further separated between males and females. Last mentioned difference is pointed out by blue and red colored dots for each box plots and the respective sex is written above the box plots. Further sampling days are indicated by the darkening of the colored dots within male and female data sets and ranked from day 7 to 14 and lastly to day 28. As seen in Figure 19, T1 was barely detected and if mostly in females than males but within the detected concentration T1 seemed to be coherent between strains. For analytes T4, T3, rT3, and T2 higher concentrations were detected in male F-344 rats than in female F-344 rats. Overall, that is a common difference regarding T4 concentration in rat plasma for which the tendency of higher concentration in males than females was also reflected in the other strains. This pattern was also clearly visible for rT3 concentrations in each rat strain. Any fluctuation between the sampling days as for example as seen in females of Wistar Han RCC rats were commonly reflected in the concentration of all analytes. Additionally, an historic RSD was calculated based on every TH analytes average of data from previous plasma analysis of untreated adult Wistar Han (CrI:WI(Han)) rats. The resulting ranges were individually indicated for males (blue) and females (pink) in the graphs by the horizontal

5.5 TH Detection in Different Rat Strains

dotted lines. Notable was that T3 and T2 concentrations of male F-344 rats were above this range on day 14 and 28.

All in all, total TH concentrations between the rat strains were within a similar range except for male F-344 rats which seemed to be higher especially in T2 concentration. This increased concentration in F-344 males possibly explained the RSD above 20 % when considering all technical replicates from both rat strain plasma pools because combination of male and female F-344 plasma resulted in an overall higher TH plasma concentration than for the remaining rat strains.

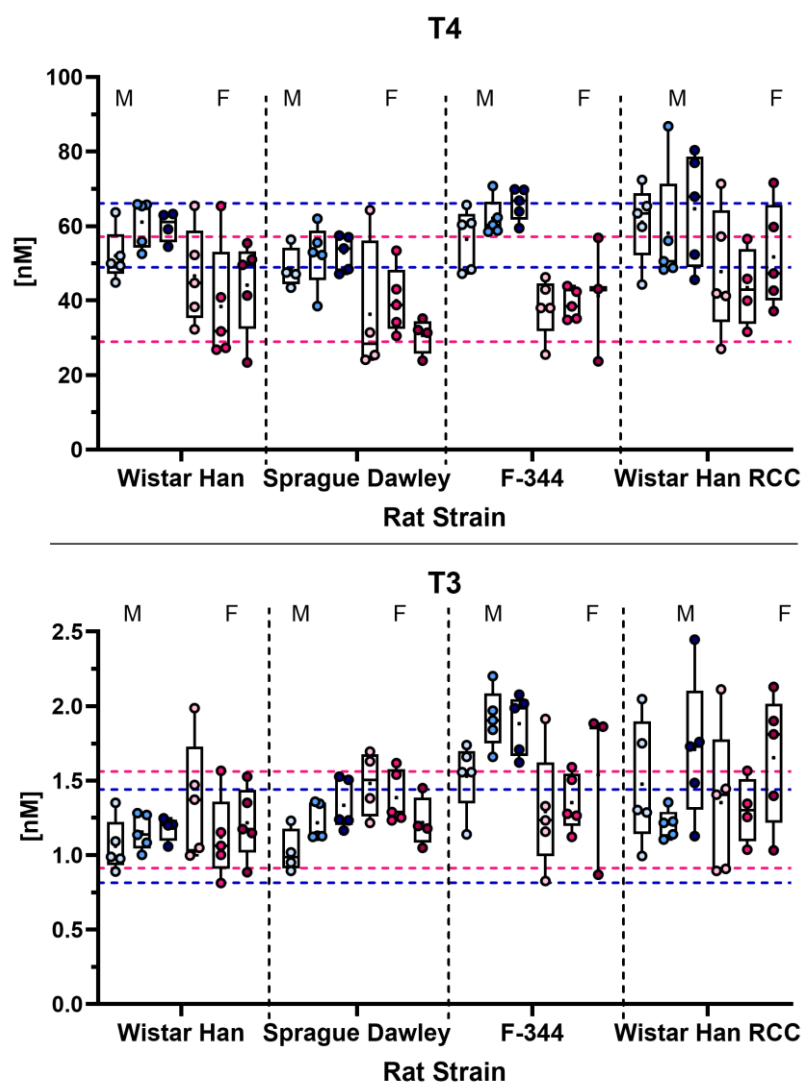


Figure 18. T4 and T3 analysis in plasma of different rat strains.

Total T4 and T3 concentrations are shown in [nM] and results are depicted pairwise (males & females) for each strain which are listed on the x-axis and the separation of the rat strains is highlighted by black dotted lines. Data sets from males and females are additionally written above the box plots and distinguishable by the color of the dots for which blue refers to males and red to females. Each data set consists of three box plots depicting three sampling days (day 7, 14, and 28), this is further emphasized by darkening of the colored dots. An RSD of over 100 historic untreated adult Han Wistar rat plasma samples was calculated. The calculated range of the historic control data is indicated by the dotted lines whereas the color blue and red refer to the range for males and females respectively.

5.5 TH Detection in Different Rat Strains

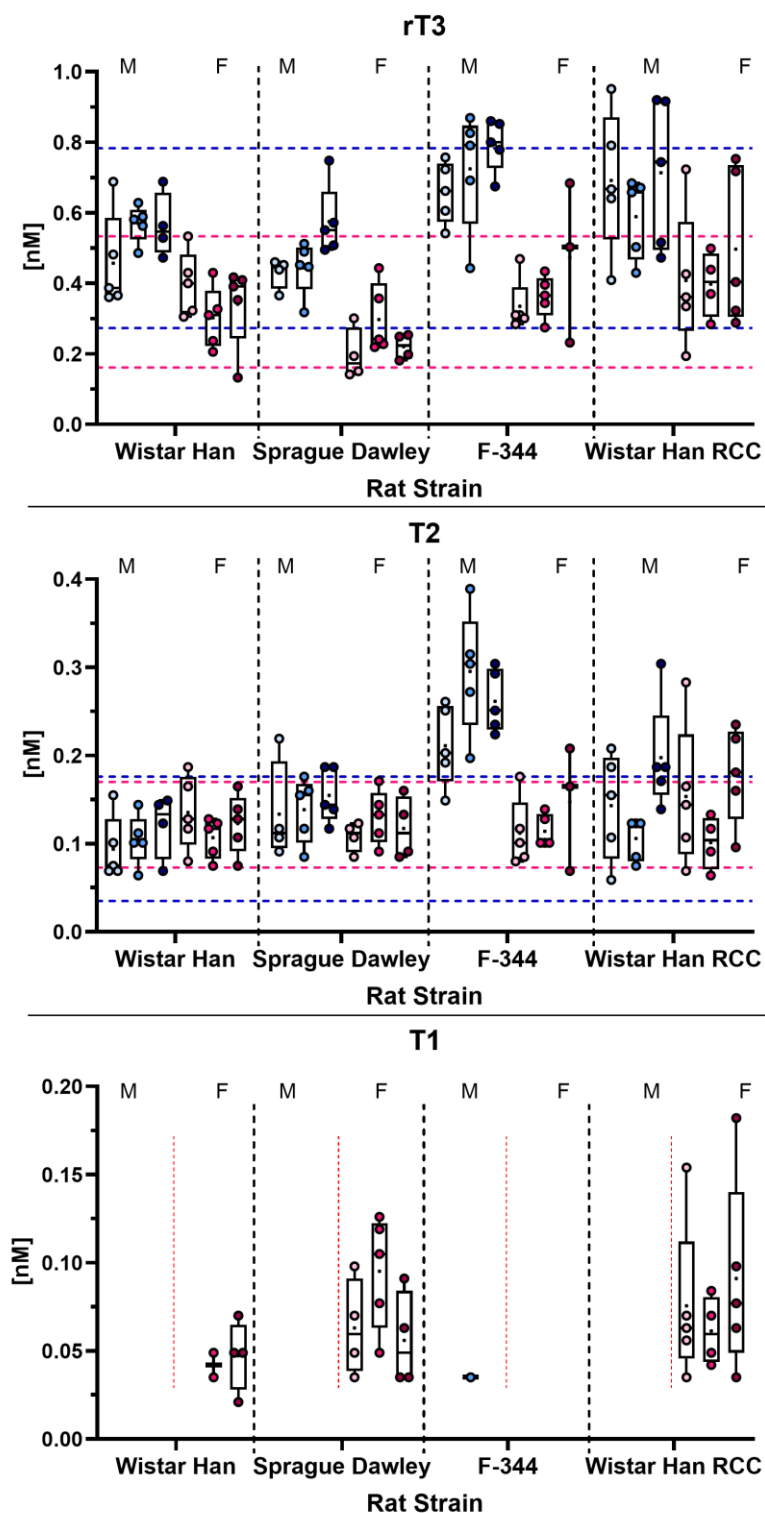


Figure 19. rT3, T2 and T1 analysis in plasma of different rat strains.

Total rT3, T2 and T1 concentrations are shown in [nM] and results are depicted pairwise (males & females) for each strain which are listed on the x-axis and the separation of the rat strains is highlighted by black dotted lines. Data sets from males and females are additionally written above the box plots and distinguishable by the color of the dots for which blue refers to males and red to females. Each data set consists of three box plots depicting three sampling days (day 7, 14, and 28), this is further emphasized by darkening of the colored dots. An RSD of over 100 historic untreated adult Han Wistar rat plasma samples was calculated. The calculated range of the historic control data is indicated by the dotted lines whereas the color blue and red refer to the range for males and females respectively.

5.6 TH Concentrations Detected at Various Ages in Control Rats

5.6.1 Simultaneous TH Changes in Rat Plasma and Whole-Brain

THs in brain tissue are not typically analyzed yet and therefore TH concentrations in control rats at study-relevant timepoints were assessed first (PND4 & PND21). In addition to the results in offspring rats, TH results from adult rats were added to evaluate the dynamic biological fluctuation of TH concentrations during development. Figure 20 represents total TH concentrations in rat plasma at ages PND4, PND21, 97-99 day-old and in dams on PND4 and PND21. Blood sampling of dams was conducted within the first study at the same days as their respective offspring, but no brain samples from dams were obtained. As indicated above but also indicated by red solid lines, the samples were received from different studies. In all figures shown in this section the different symbols refer to different ages such as triangles symbolize PND4, dots PND21, squares adult rats (97-99 day-old and 71-72 day-old), and stars dams as well as their blue and red color signify either males or females. In plasma, age-dependent concentration patterns were detected for analytes T4 and T3. These patterns had the lowest concentrations on PND4 which strongly increased on PND21 in a similar manner for both sexes. T4 and T3 concentrations in adult rats and dams were all in all lower than on PND21. T3 in male adults was similar to female adults, and for T4 the typical discrepancy between males having higher concentration than females was detected. This described discrepancy was also detected for analyte rT3, but remaining analysis was unchanged between the ages same as for T2.

5.6 TH Concentrations Detected at Various Ages in Control Rats

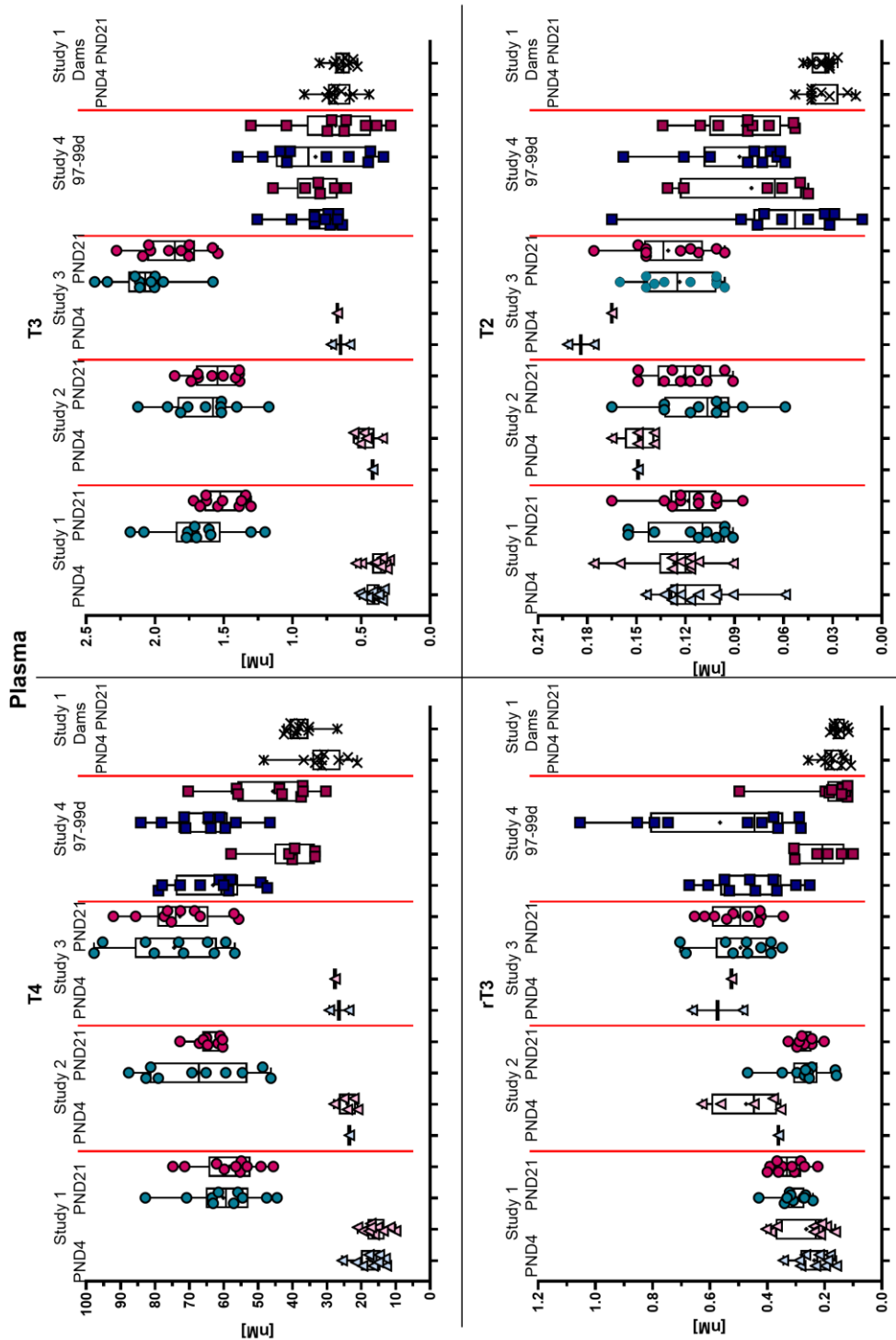


Figure 20. Total TH concentrations in rat plasma of control animals examined at various ages. Graphs are ranked from T4 and T3 in the top row to rT3 and T2 in the bottom row. The male and female data sets from each study are presented as box plots whereas the respective study as well as age is stated above the box plots. Each data set per age consist of males and females which are distinguishable by the either blue or red colored symbols. For a quicker visual separation of age, the symbols per age group were selected differently: triangles refer to PND4, dots to PND21, squares to 97-99d (adult), and stars to dams. Blood sampling from dams from study 1 was done at the same days as for their pups meaning on PND4 and 21 which is described in the graph. Red solid lines further partition the different study data sets.

5.6 TH Concentrations Detected at Various Ages in Control Rats

Besides plasma samples, whole brain samples were received from the same animals and analyzed for their TH concentrations. Figure 21 demonstrates the results from TH analysis in rat whole brain with the same legend as previously described. Unlike plasma analysis, rT3 and T2 did show age-dependent changes such as rT3 concentration peaked on PND4 and decreased from there on until adult age. T3 and T2 on contrary yielded low concentrations on PND4 and in adult rats but peaked on PND21 similar to T3 pattern in rat plasma. T4 concentration was as well similar on PND4 and in adult rats but showed the largest variation on PND21. T4 concentration on PND21 varied between studies for which in the first study the PND21 concentration was higher than on PND4 but in the second study the concentration was alike. This fluctuation on PND21 for T4 was not detected in rat plasma of the same studies. Lastly, the data sets from both matrices of study 1, 2 and 4 were contrasted against each other for a better overview of changes in TH concentrations at different ages as well as in plasma and brain. These resulting dot plots for each analyte are summed up in Figure 22. The symbols like in the other figures refer to PND4 as triangles, PND21 as dots, and adult rats as squares as well as blue and red color indicate male and female data points. The top row includes graphs of T4 on the left and T3 on the right. For these two analytes the graph visualized that their concentration on PND4 was the lowest on plasma level (bottom vertically) but it was similar to adult rats on brain level (left horizontally). A clear separation of PND21 data on each axis was detected for T3 and on brain level for T2 as well which was indicated by red lines. As seen in Figure 20, rT3 and T2 concentrations were unaffected in plasma over the examined ages which was also emphasized in Figure 22 since on plasma level (y-axis) all data points were alike except for a couple of adult rat data points. Horizontally, an increase of rT3 concentration in brain with decreasing age was visualized and for T2 in brain, a separation between all age groups was detected as well.

5.6 TH Concentrations Detected at Various Ages in Control Rats

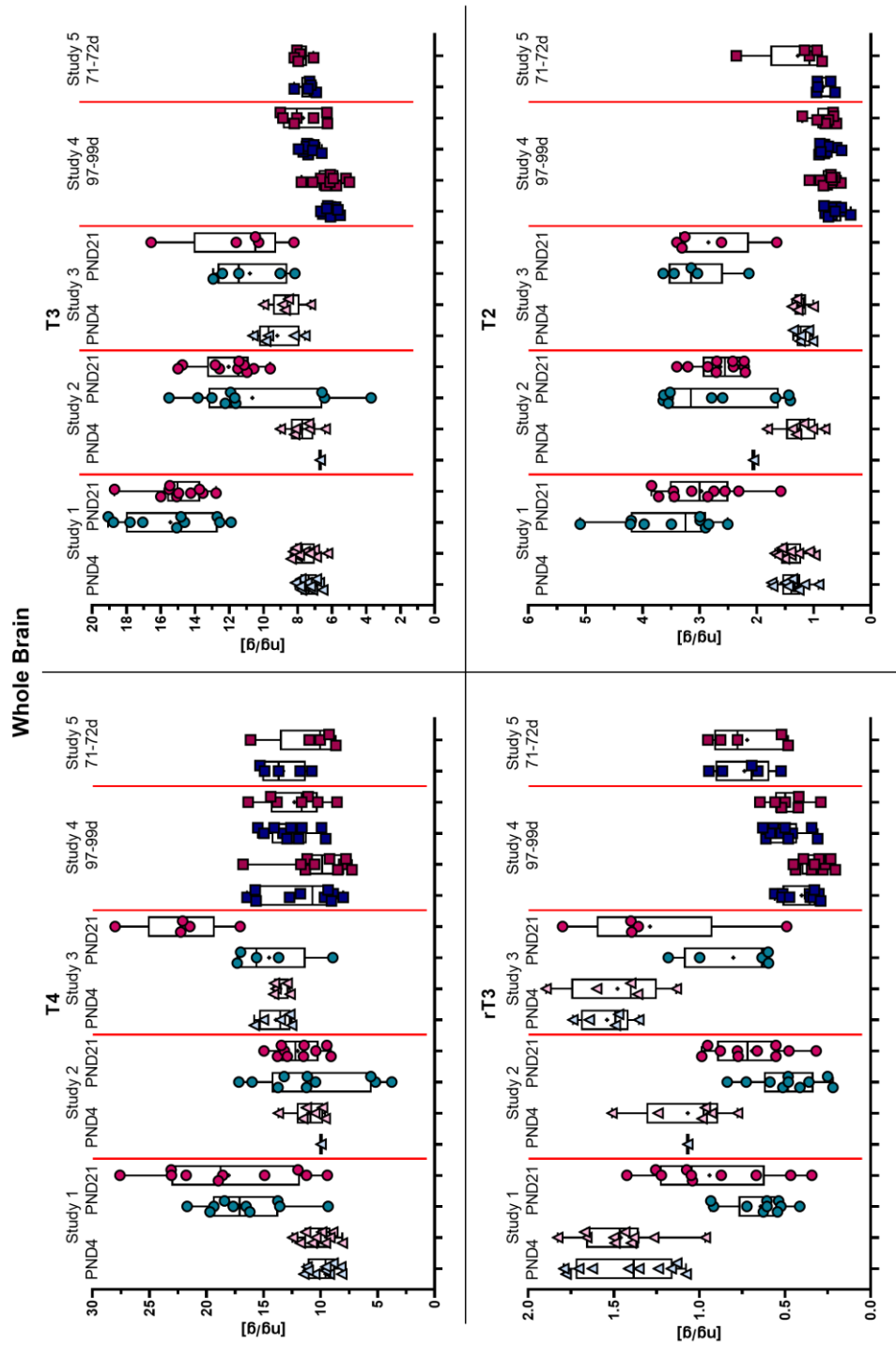


Figure 21. Rat whole brain TH analysis in rats of different ages. Graphs are ranked from T4 and T3 in the top row to rT3 and T2 in the bottom row. The male and female data sets from each study are presented in a box plot whereas the respective study as well as age is stated above the box plots. Each data set per age consist of males and females which are distinguishable by the either blue or red colored symbols. For a quicker visual separation of age, the symbols per age group were selected differently: triangles refer to PND4, dots to PND21, squares to 97-99d (adult), and stars to dams. Blood sampling from dams from study 1 was done at the same days as for their pups meaning on PND4 and 21 which is described in the graph. Red solid lines further partition the different study data sets.

5.6 TH Concentrations Detected at Various Ages in Control Rats

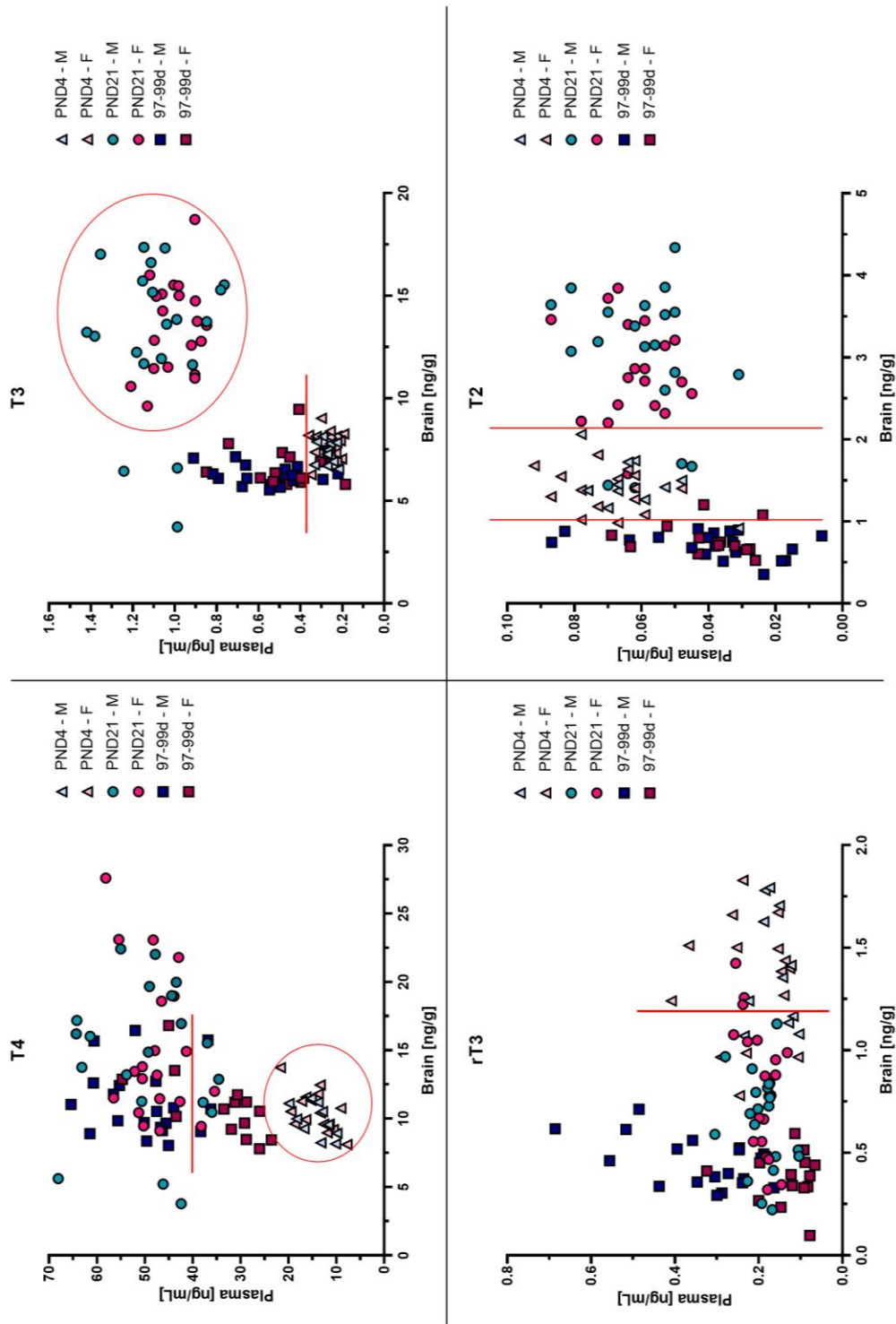


Figure 22. Contrasting plasma to whole brain TH concentrations of PND4, and PND21 pups and of adult rats. The graphical order starts from the top left with T4 and ends at the bottom right showing T2 results. TH values from PND4, PND21, and from adult rats of the previous shown plasma and whole brain measurement are plotted against each other. Plasma concentrations are found on the y-axis and brain on the x-axis. Every individual data point for both matrices was received from the same animal. The various symbols define a certain age: triangles PND4, dots PND21, and squares 97-99-day old also refer to as adult rats. Blue and red color further distinguish between males and females. Tendencies of partitioning of certain age groups are highlighted by red lines or circles.

5.6 TH Concentrations Detected at Various Ages in Control Rats

In conclusion, there is an age-dependent change of TH concentrations in both matrices as previously described. The age-dependent pattern differed for each analyte as well as matrix such as that changes were most prominent in each matrix for T4 and T3 whereas for rT3 and T2 only in brain. For a better overview of this conclusion, a summary figure of the data showing the mean with SD per age, sex, and matrix but combining concentration of the analytes was inserted below (see below Figure 23).

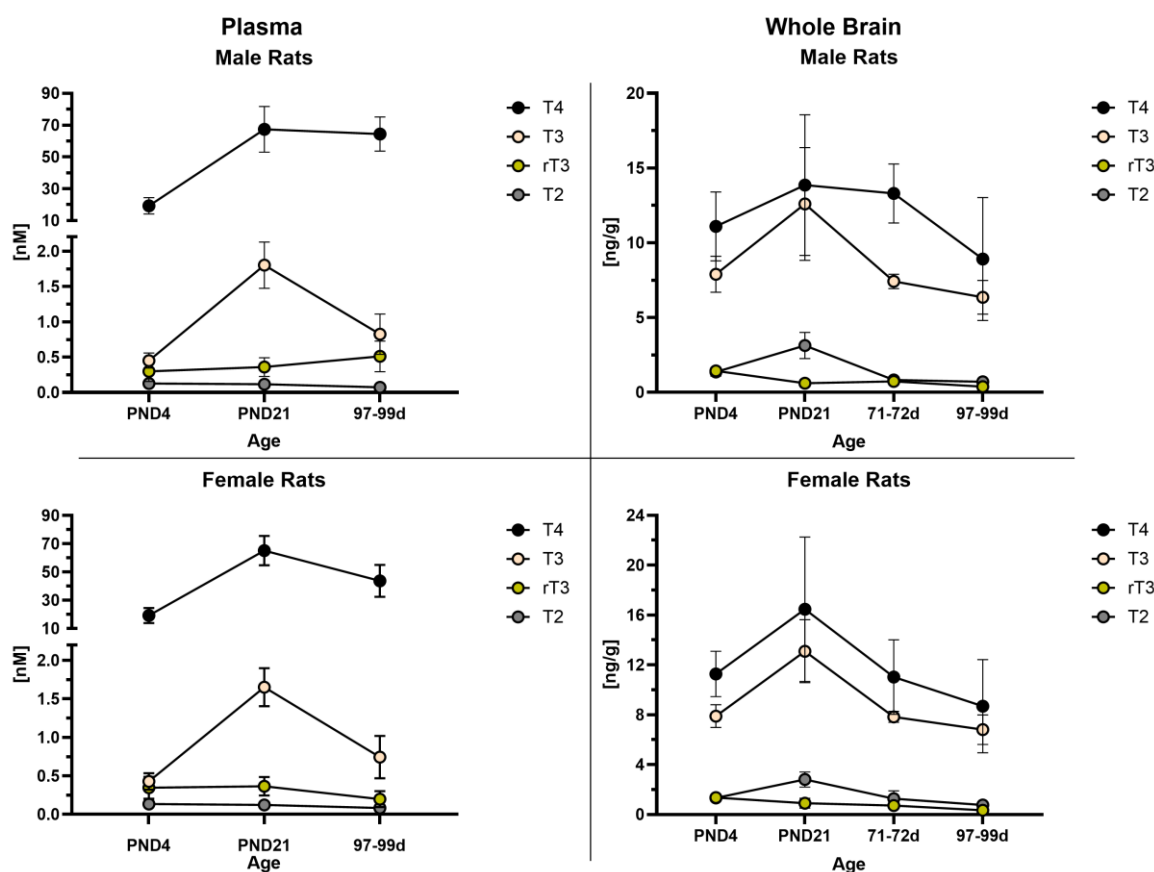


Figure 23. Summary figure of total TH concentrations in rat plasma and whole brain at specific ages.

The graphs are separated by sex and matrix and each graph contains detected concentrations of all analytes. All values per age and study which data were above shown separately by study were combined to better demonstrate the age-dependent change of TH concentrations. Thus, in each graph the mean with standard deviation (SD) per age is depicted. Analytes are distinguishable by color. Black dots identify T4, light orange T3, green rT3, and grey T2.

5.6 TH Concentrations Detected at Various Ages in Control Rats

5.6.2 Rat Brain-Region Specific TH Changes During Development

Besides whole brain TH analysis in offspring and in adult rats, region-specific TH analysis was performed in cerebellum and in the remaining cortex. As for whole brain analysis control samples were received from different studies and analyzed regarding their TH concentrations. In Figure 24 TH concentrations in cerebellum and cortex at different ages are portrayed wherefore the examined age was written above the data which was pairwise depicted for males and females (x-axis). TH concentration found in cerebellum is symbolized with a dot and cortex with a triangle. Analyte T4 is summed up on the top left corner in the mentioned figure and it displayed regional similarity in all ages. The graphs from T3 and T2 are placed on the right side on top of each other in which age-dependent regional alterations were visible. Regional diversity was noticed with low concentration in cerebellum compared to cortex on PND4. On PND21 and in adult rats the diversity between the regions was balanced out, but all in all the highest concentration was found on PND21. In the bottom left corner results for rT3 showed regional similarity on PND4 as well as overall the highest concentration on PND4 compared to the other ages. Regional similarity was retained in adult rats, but on PND21 rT3 concentration in cerebellum was detected to be lower than in cortex.

All in all, TH analysis in specific brain regions did show dissimilarity at certain ages but also similarity which both were analyte specific.

5.6 TH Concentrations Detected at Various Ages in Control Rats

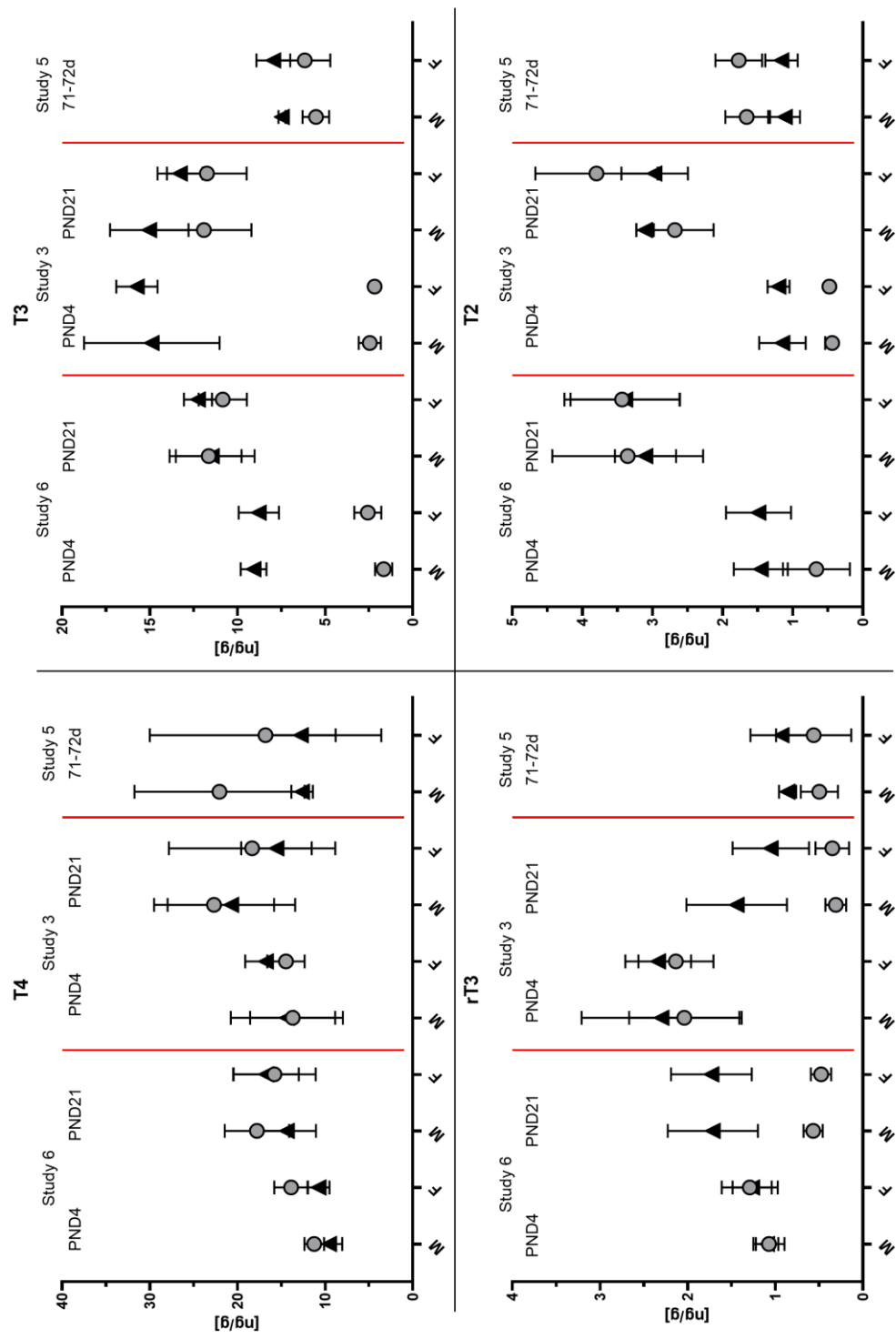


Figure 24. TH concentrations assessed in specific brain regions on PND4, 21, and in adult rats. Each graph illustrates the concentration [ng/g] on the y-axis for male and female rats from different studies on the x-axis. The first row contains graph of T4 and T3, and the second row of rT3 and T2. The red solid lines separate the data from different studies whereas the first two studies involved PND4 and PND21 offspring and in the last studies adult rats were examined. Data depicted as grey dots indicate the concentration measured in cerebellum and the black triangles the concentration in cortex.

5.6 TH Concentrations Detected at Various Ages in Control Rats

5.6.3 TH Analysis of Formalin-Fixed and Paraffin-Embedded Half-Brain

Method applicability was further investigated by a first test trial to extract THs from formalin-fixed (FF) and paraffin-embedded (PE) half-brain from adult rats (n=2) which would further expand the possibility for retrospective TH analysis of tissue. From each rat brain, one half was prepared with a different tissue fixation meaning either embedded in paraffin or fixed in formalin.

First testing of preparation is described in detail in section 4.1.3. The results showed no detection of THs in FF replicates, however analytes peaks were detectable in the samples from PE half-brain. To ensure that the amount of weighed in powder was not insufficient for the detection of TH peaks for FF half-brain analysis, the whole remaining powder was prepared and analyzed (127.92 mg). The same was conducted for PE half-brain yielding around 189.77 mg. Additionally, a total volume of 1500 μ L 1 % FA in ACN (v/w) was added to extract THs and two 3 mm steel balls were inserted into the 5 mL tube. Homogenization and extraction of the sample was performed on a Bead Ruptor Elite (biolab products, Bebensee, Germany) as previously conducted for pulverization of the rat brain tissue. This procedure led to a homogenization of the PE half-brain pieces. The subsequent sample preparation was conducted as described under 4.1.2. Results showed similar detection of T4 and T3 concentration in PE half-brain samples, however improvement was achieved by detecting T4 and T3 in FF tissue after weighing in a higher amount as seen in Figure 25. The calculated concentrations of TH analytes were all in all too low compared to the expected concentration in adult rats which was around 12 to 20 times higher.

For the second testing, the procedure was modified as mentioned in section 4.1.3 which basically consisted of a stepwise washing procedure and an incubation overnight in Millipore H₂O to ensure rehydration of the tissues. Technical replicates of 60 mg or 30 mg of FF half-brain (n=4) were weighed in for TH analysis. PE half-brain was weighed in completely (around 354 mg), 3.2 mL 1 % FA in ACN was added followed by homogenization and extraction using Bead Ruptor Elite as stated above. This generated a suspension which was centrifuged, and four replicates of the supernatant were removed (around 550 μ L, n=4). The subsequent sample preparation was handled as described in 4.1.2. As presented in Figure 25 under “2nd Trial” lower T4 and T3 concentrations were detected in PE half-brain compared to the results under “1st Trial”. T4 and T3 concentrations detected in FF half-brain were slightly higher than after the first testing procedure, however T3 was not detected in all replicates. In addition, rT3 was detected in FF half-brain (around 0.3 and 0.15 ng/g) whereas T2 was not detected in any of the

5.6 TH Concentrations Detected at Various Ages in Control Rats

samples from the second testing procedure. Total number of technical replicates prepared and analyzed were four, but mostly in two out of four replicates were the analytes identifiable.

All in all, mostly T4 and T3 were detectable after either of the testing procedures, however concentrations were 12 to 20 times lower than expected for T4 and T3 in brain and for rT3 around two to three times lower. Thus, these testing procedures were not sufficient for TH analysis of fixed tissue.

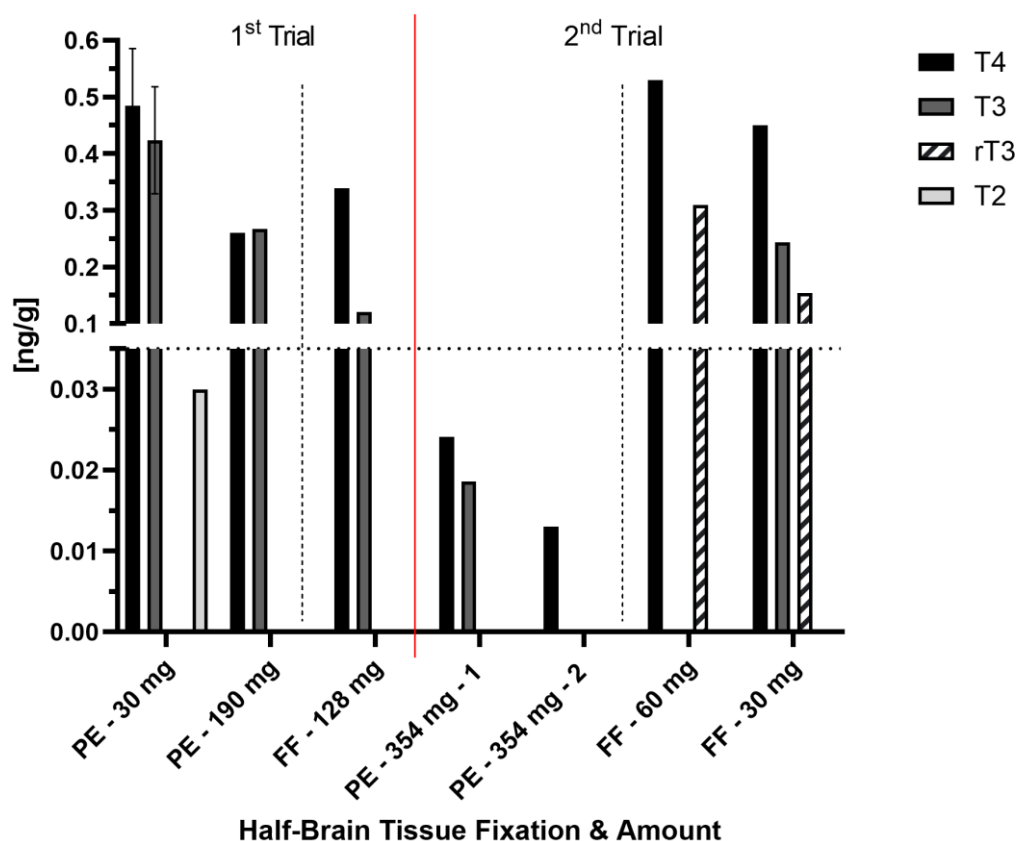


Figure 25. TH concentrations detected in paraffin-embedded (PE) and formalin-fixed (FF) half rat brain. On the x-axis the different fixation methods and the corresponding sample weight for preparation are named. The different colored bars refer to the detected TH analytes whereas black refer to T4, dark grey to T3, striped bar to rT3, and the light grey bar to T2. The dotted black lines partitions the fixation methods whereas the solid red line divides the different testing procedures which is further written above the bars. The first bar contains three values for T4 and T3 whereas all other bars one value. PE half-brain during the second trial was completely weighed-in, homogenized and extracted in 1 % FA in ACN using Bead Ruptor Elite and four technical replicates (around 550 μ L) of the supernatant of the centrifuged suspension was removed for preparation.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

The TH system can be influenced by substances through direct or indirect mechanisms. Prominent examples of substances directly affecting the TH system are PTU, Ethylene Thiourea (ETU), and Methimazole (MMI). PTU and MMI are thyreostatic drugs which are prescribed to treat against hyperthyroidism. On contrary, ETU is a metabolite of ethylene bisdithiocarbamates (EBDTCS) which are employed as fungicides. All three compounds have in common that they interfere directly with the TH system by inhibiting TPO in the thyroid gland leading to a decreased TH synthesis and subsequent lowered TH concentrations in plasma. Out of the three, especially PTU is commonly used as a positive verification control in THD method development because of its strong inhibitory effect. One further investigated substance representative for direct MOAs was Phenyl Isothiocyanate inhibiting NIS. Indirect mechanisms that affect circulatory TH concentrations as a secondary cause are e.g. liver enzyme induction or the competitive binding to plasma TH-binding proteins. The substances that were analyzed representing indirect TH mechanisms were the polychlorinated biphenyls (PCB) Aroclor 1254 and the fungicide Vinclozolin as well as 3,3',5,5'-Tetrabromobisphenol A (TBBPA) and Perfluorohexanesulphonic Acid (PFHxS) which last two mentioned substances competitively bind to the plasma TH-binding proteins. Therefore, the alterations of total TH concentrations in plasma caused by direct or indirect MOA will be compared and in detail shown in this section. Information about animal number and dosage are enumerated in Table 10. Additionally, each graph in sections 5.7.1 and 5.7.2 is plotted similarly meaning that results in male rats were presented in the first column and next to it in female rats. The dots of the box plots were colored in either blue or red indicating either males or females. The increase darkening of color further signified the increase in the given dose meaning control to HD (high dosage) group. The dosage groups and the respective sampling days were plotted on the x-axis and the y-axis presented detected concentration [nM] in plasma.

Due to internal past extensive *in-vivo* research on different insecticides operating through different MOAs, various plasma and brain samples from adult rats from these studies were available. Since the assessment of THD is an important part for the regulatory assessment of e.g. insecticides, analysis of the mentioned adult rat plasma and brain samples was conducted to assess their influence on TH concentrations in both matrices. THD by insecticides is suspected to be a combination of various MOAs such as some of the suspected TH-related MOAs are the inhibition of TH synthesis, binding to its TR, competitive binding to transport proteins in blood or the induction of TH enzymes. The insecticide classes were Pyrethroids

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

(substances A-C), Organophosphates (substances D-F), Organochlorines (substances G and H), and lastly Phenyl Pyrazoles and Organotin compounds (substances I-K). Animal samples from studies with 7-day exposure were available for substances A, C to K and samples after 28 days for substances B-C, G, and I. The longer exposure period study entailed a LD (low dose) as well as a HD group and extra plasma sampling days which were conducted on day 7, 14 and 28 which plasma samples were thus available for compounds B-C, G, and I. The HD was selected to be in accordance with the maximum tolerable dose and one dose below was selected which is referred to as LD. All received samples are tabularized in Table 12. All graphs containing plasma results were mainly constructed in a similar pattern showing males and females results either blue or red colored. Dosage groups were listed on the x-axis and control to HD groups are further marked by darkening of the respective color. Plasma concentration [nM] is numbered on the y-axis. The red solid lines highlighted separation between different study results whereas the different sampling days were partitioned by dotted lines. The graphs entailing analysis in adult rat whole brain differ from the mentioned legend that no LD samples were available and thus results from the given HD groups were colored in grey or black. The box plots encompassing control group results were still highlighted in either blue (males) or red (females). Detected concentration in brain [ng/g] was plotted on the y-axis and control groups and the blinded substances on the x-axis. Like plasma results, different study results were separated by red solid lines. Results from males and females were divided by dotted black lines. Sampling days as well as sex were additionally written above the box plots in graphs for both matrices. Following in this section the TH alterations in plasma and whole brain of rats exposed to various pesticide classes will be described in detail.

5.7.1 Direct TH-Related MOAs

5.7.1.1 Effect of TPO Inhibition on Total TH Concentrations

One direct thyroid-related MOA is the inhibition of TPO in the thyroid gland preventing TH synthesis. Exemplary substances tested during this project were the thyreostatic drugs PTU and MMI and the metabolite ETU of EBDTCs fungicides. PTU and MMI were administered by gavage in doses of either 1 or 10 mg/kg bw/d and 25 or 100 mg/kg bw/d, respectively. ETU was given in the diet in concentrations of 75 or 300 ppm which equal doses of around 6 or 24 mg/kg bw/d assuming a body weight of 250 g and a food consumption of 20 g per day.

Results for detected total TH concentration after exposure to PTU in rat plasma are demonstrated in Figure 26 and Figure 27. Overall, PTU exposure led to a strong decline of all TH analytes after no later than 14 days which was already profound for T4 in males from the LD group (decline of around 90 %). After 14 and 28 days total T4 concentrations in the HD

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

groups decreased up to 97-99 %, total T3 up to 78-90 %, and total rT3 around 84-92 % in both sexes. Notable was the initial increase in total rT3 concentrations in the HD group of female rats which was more than double the mean control value at day 7. In the following samplings days rT3 concentrations were significantly decreased in female rats of the HD group. Furthermore, no significant effect was determined in total rT3 concentrations in females of the LD group in neither of the sampling days which on contrary were significant changed in males at days 14 and 28. In males and females total T2 concentrations were not detectable anymore at day 28. The strong decline of total T4, T3, and rT3 was similar after exposure to MMI which concentrations were reduced around 82-95 % in the LD groups and 94-99 % in the HD groups of both sexes at day 14 (see Figure 28 and Figure 29). No control plasma samples were available at day 28, however LD and HD groups at day 28 yielded similar results as at day 14.

Compared to PTU and MMI, ETU affected total TH concentrations differently which is illustrated in Figure 30 and in Figure 31. The overall reduction of total T4 and rT3 reached their maxima by around 56 % and 62 % in male rats. In female rats maximum T4 decline was around 47 % and rT3 was not significantly altered. Opposed to a decline of T3 like for the other substances, ETU exposure caused an increased T3 concentration above the control group by up to 57 % on day 7 for males, and time-dependently in females up to 139 % on day 28. As indicated in Figure 30 the analyte T3 increased in the HD group of males and females at most sampling days. A statistically significant increase in the LD group was only detected after 28 days in males and females. The same pattern was further reflected in T2 concentration which in particular significantly increased over time in the HD of female rats as seen in Figure 31.

As expected, the investigated TPO inhibitors revealed a strong reduction of TH concentrations in rat plasma, but potency differences between the substances were observable of which ETU caused less strongly TH reductions than PTU or MMI.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

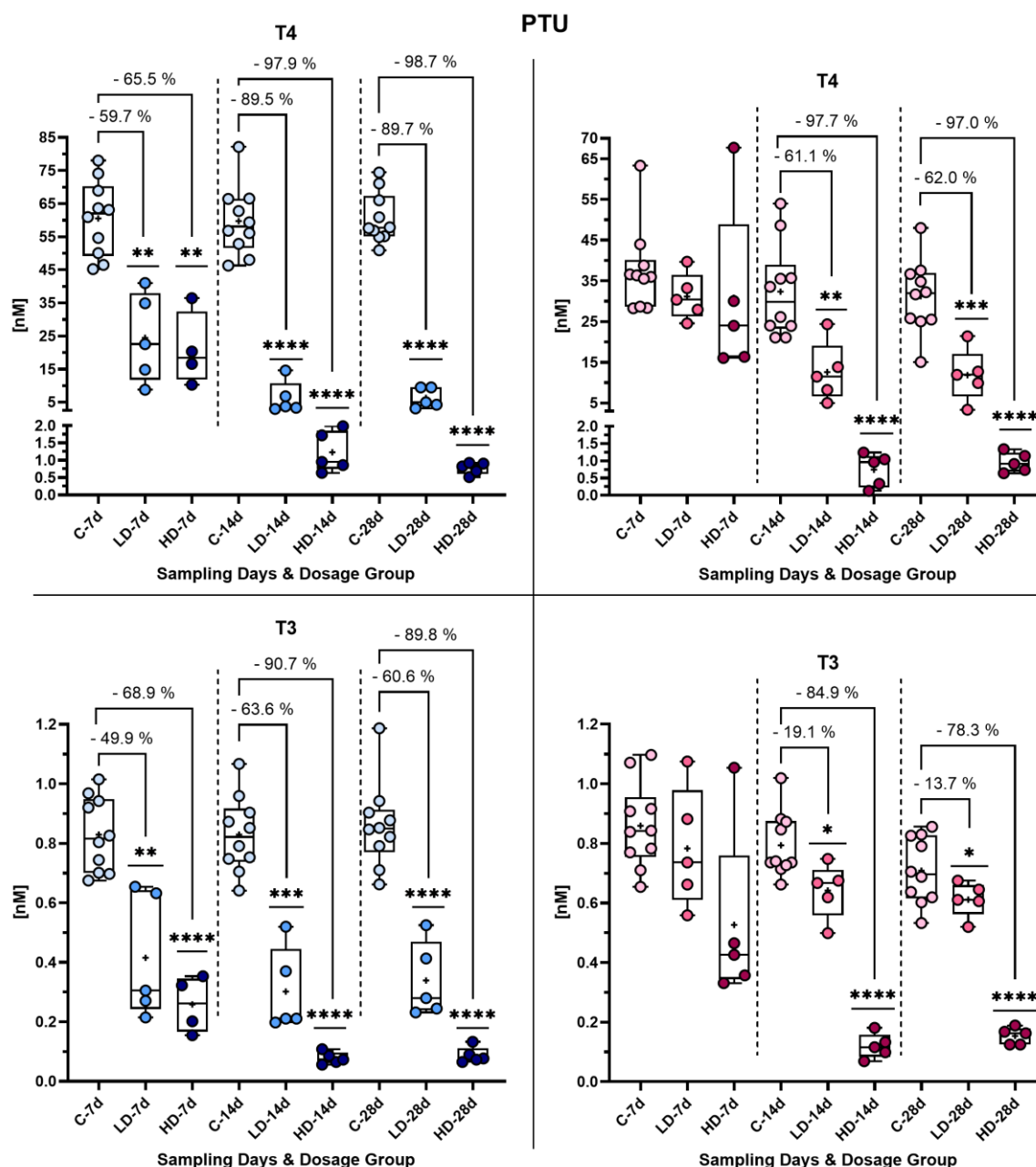


Figure 26. Total T4 and T3 concentrations at three sampling days from control and PTU-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

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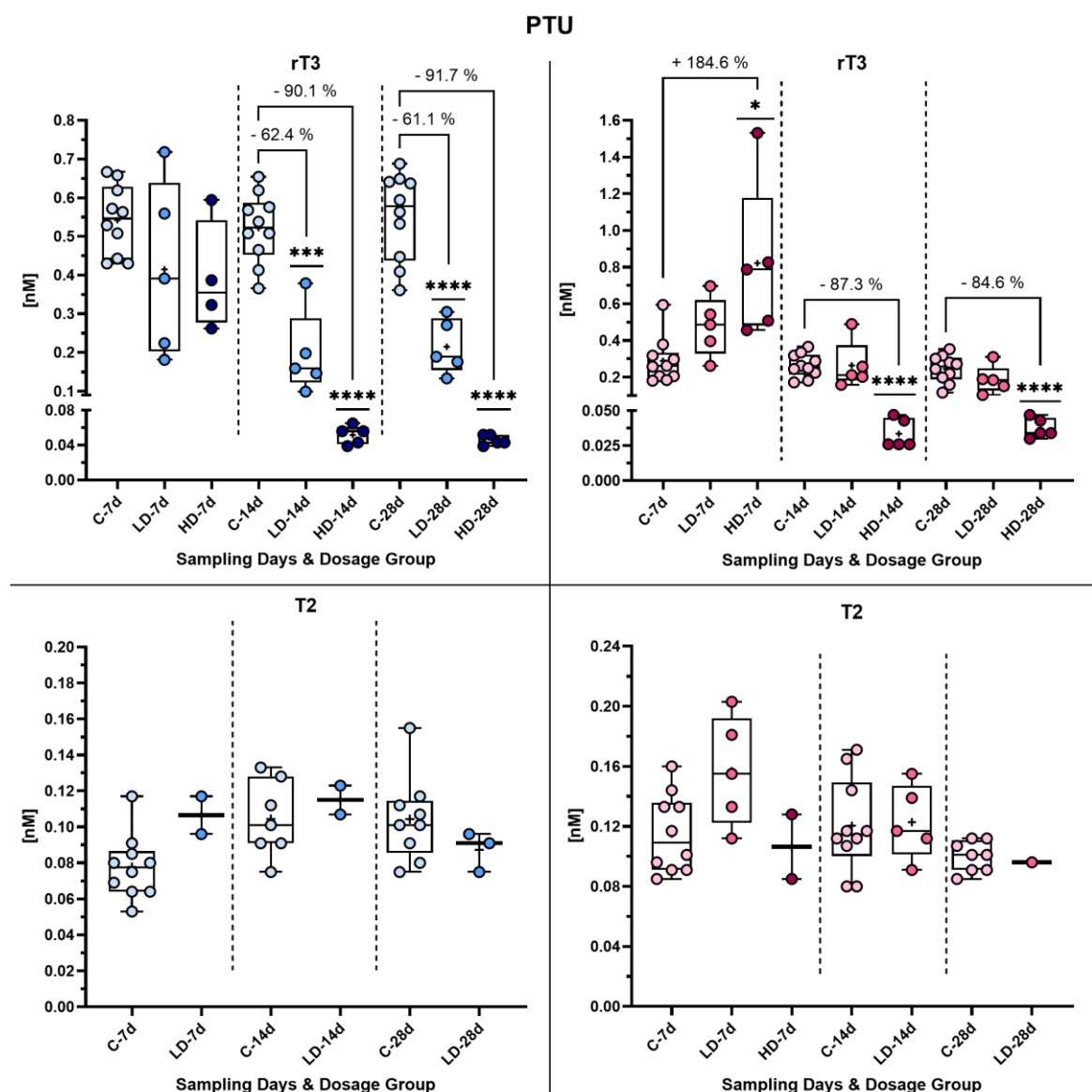


Figure 27. Total rT3 and T2 concentrations at three sampling days from control and PTU-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T2 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

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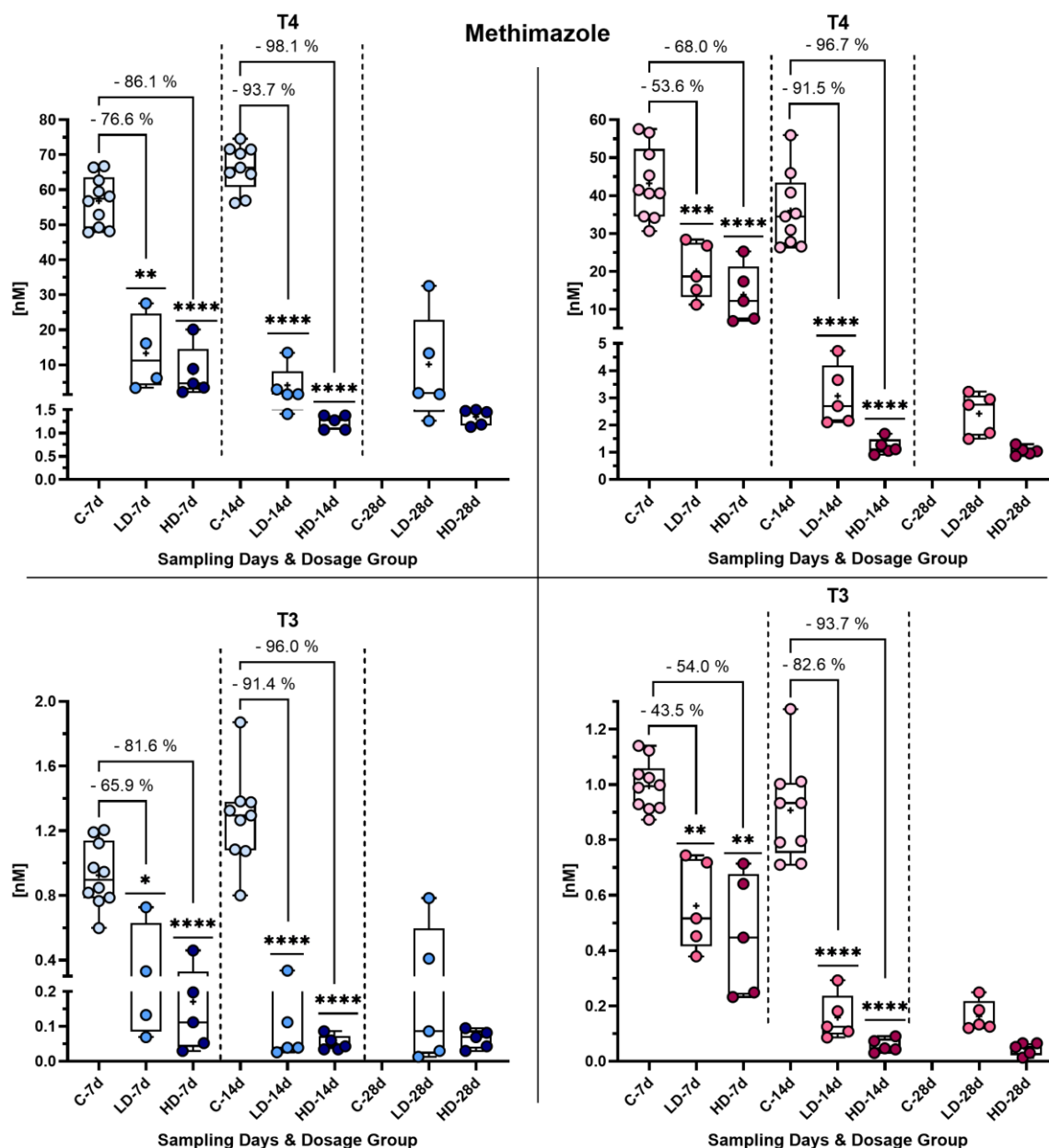


Figure 28. Total T4 and T3 concentrations at three sampling days from control and MMI-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

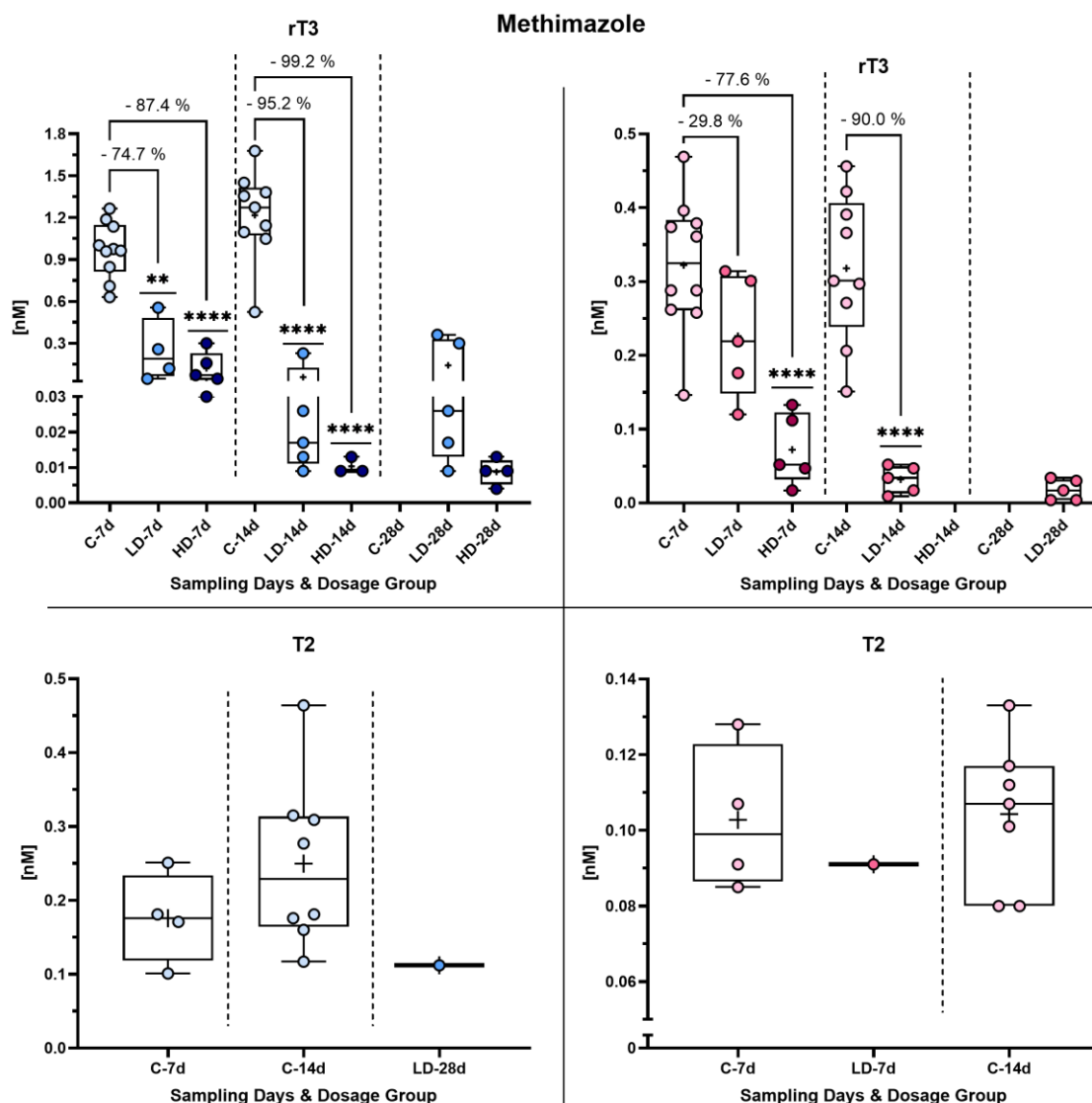


Figure 29. Total rT3 and T2 concentrations at three sampling days from control and MMI-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T2 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

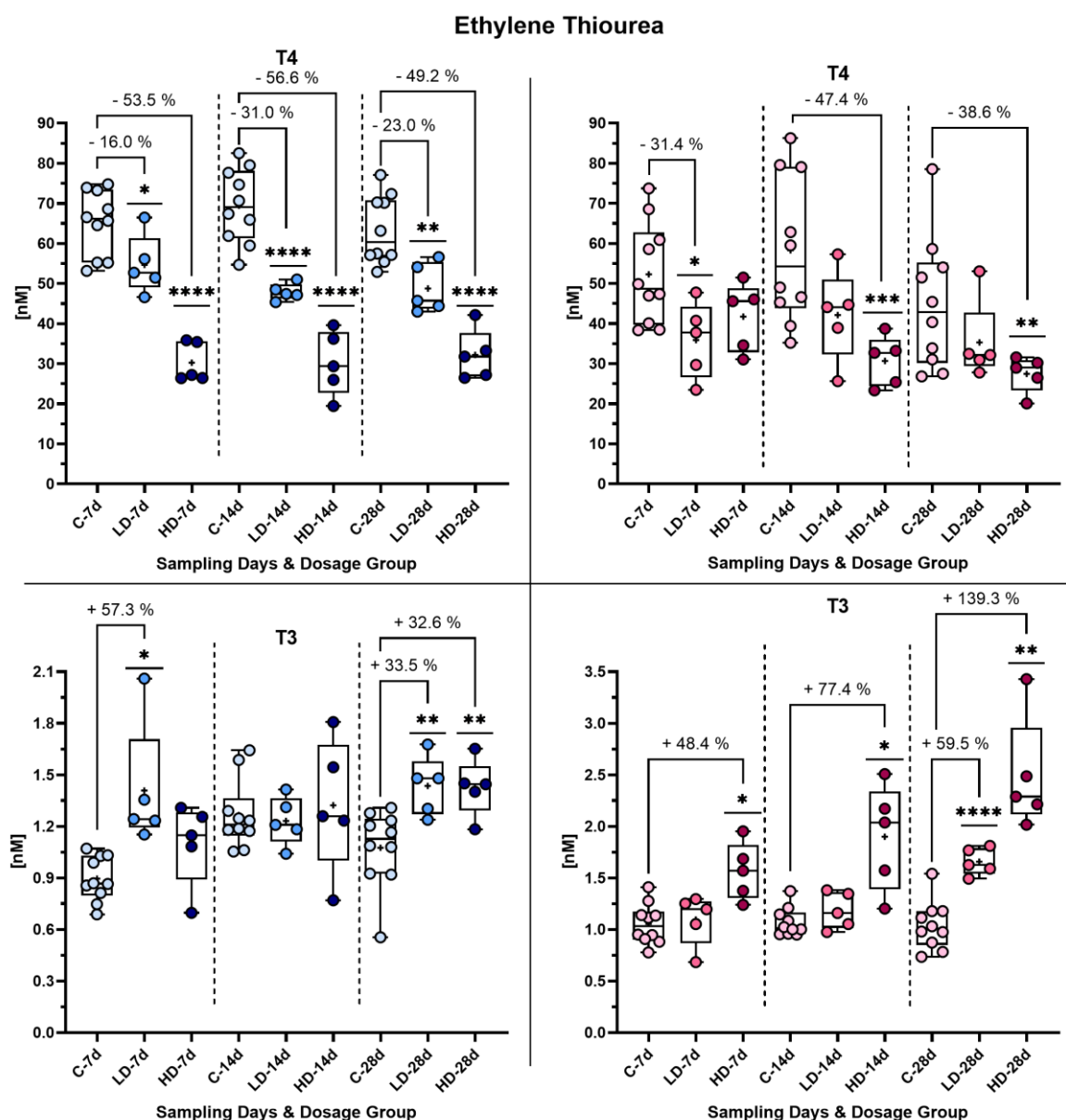


Figure 30. Total T4 and T3 concentrations at three sampling days from control and ETU-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with **T4** and on the bottom **T3** whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

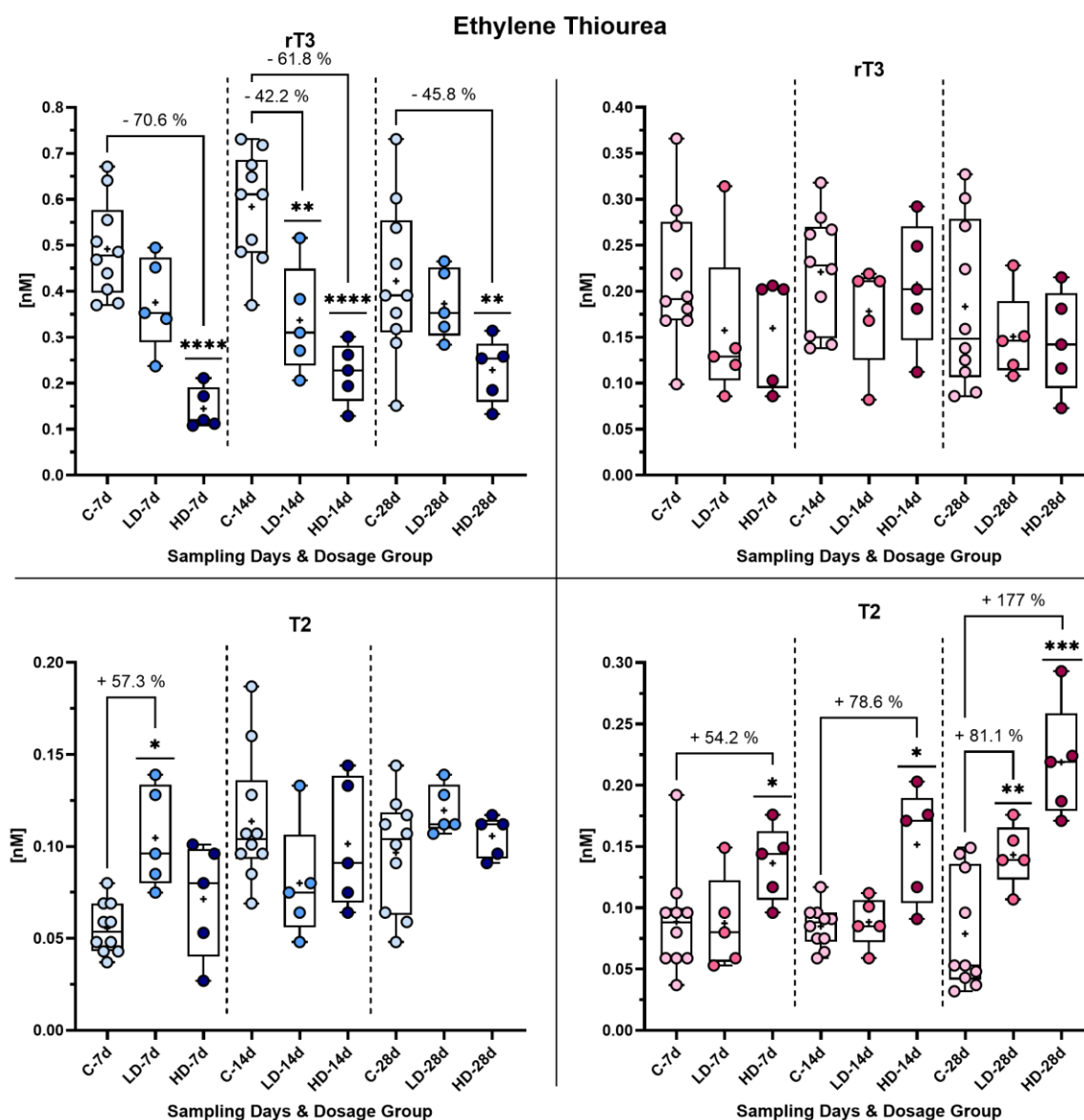


Figure 31. Total rT3 and T2 concentrations at three sampling days from control and ETU-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T2 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction is compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.1.2 Total TH Concentration in Rat Plasma after NIS Inhibition

NIS inhibiting substances block the binding site of the transporter which causes a lack of Iodide transportation into the thyroid follicular cell leading to impaired TH synthesis. An exemplary substance was tested towards its effect on total TH concentrations in rat plasma. Phenyl Isothiocyanate was administered by gavage in a dose of either 15 or 30 mg/kg bw/d. Due to severe clinical findings during the animal study, the HD of Phenyl Isothiocyanate was reduced to 22.5 mg/kg bw/d and thus no blood sampling was conducted of the HD group on day 14. The detected total T4 and T3 concentrations in rat plasma are shown in Figure 32. A notable difference between male and female rats was that mostly male rats established significant reductions ranging from 20-62 %. The initial higher HD concentration led to reductions of 62 % (T4) and 50 % (T3) at day 7 which was less strong with the adjusted HD at day 28 (42-43 %). Total rT3 concentration in males was consistently lowered by around 28-38 % in the LD animals and by around 70-72 % in the HD animals which is illustrated in Figure 33. Lastly, T2 was only significantly affected in HD animals at day 28 by 61 %. In females all analytes were for the most part unaffected by the exposure except for T4 and rT3 in HD animals at day 7 experiencing a decline of 33 % and 63 % compared to the control group accordingly.

To sum up, Phenyl Isothiocyanate reduced TH concentrations in plasma which were mostly significant altered in male and not in female rats.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

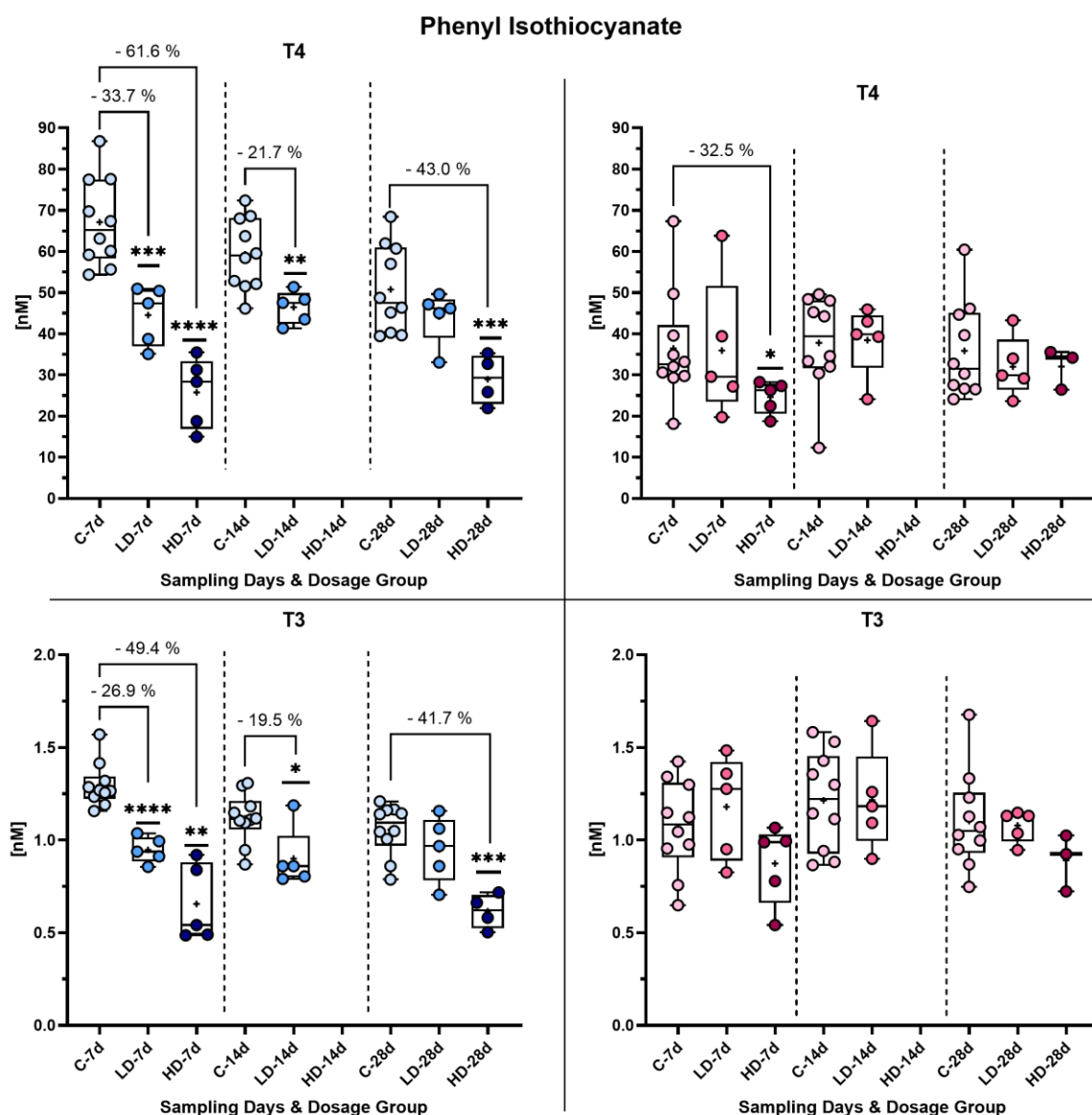


Figure 32. Total T4 and T3 concentrations at three sampling days from control and Phenyl Isothiocyanate-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

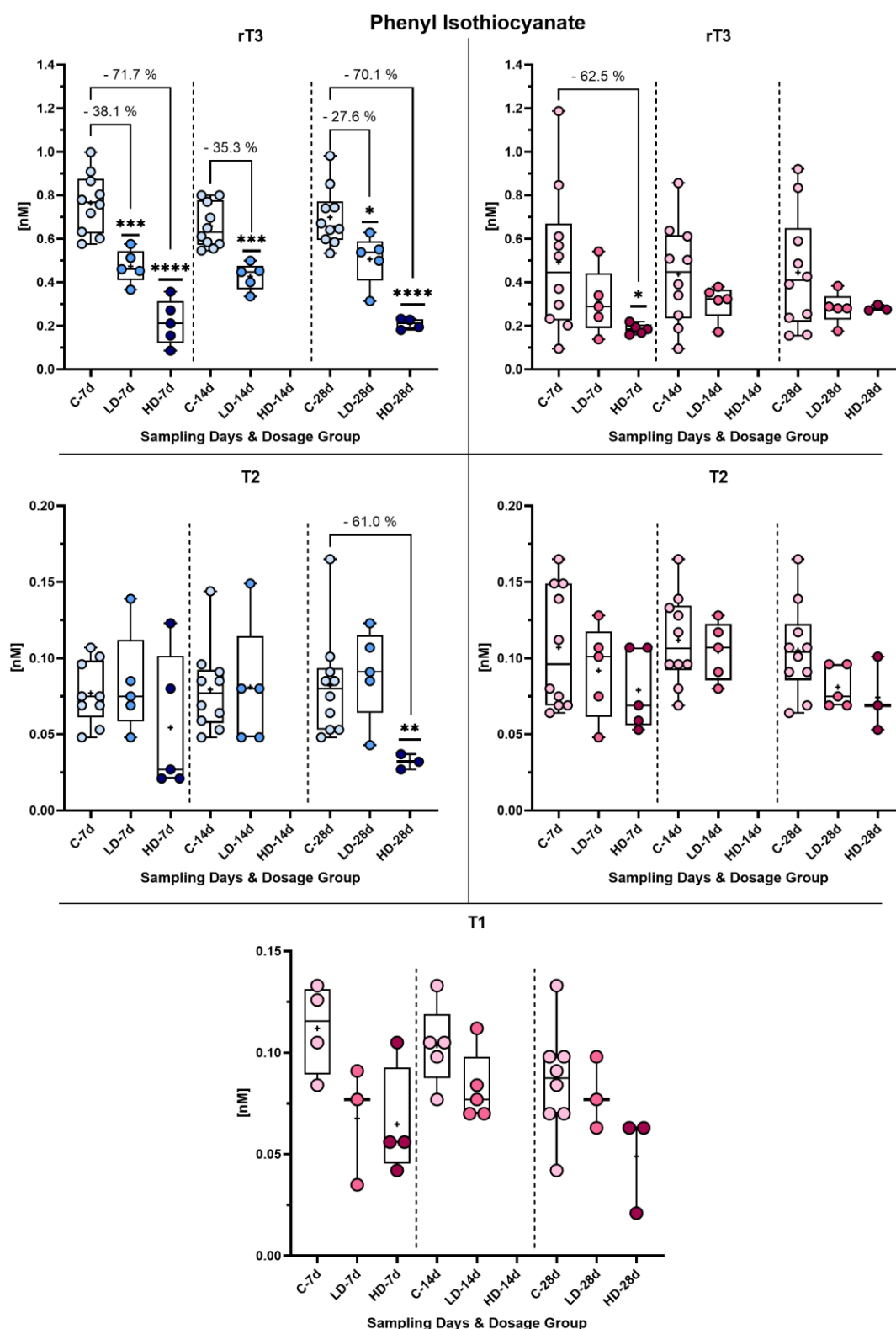


Figure 33. Total rT3 and T2 concentrations at three sampling days from control and Phenyl Isothiocyanate-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T2 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.2 Indirect TH-Related MOAs

5.7.2.1 Liver Enzyme Inducers Affect Total TH Concentrations

Liver enzyme induction causes indirectly a change in TH concentrations by the assumably increase of TH elimination due to phase II enzyme induction. During this work representatives of this possible mechanism were analyzed: Aroclor 1254 and Vinclozolin as well as the blinded substances G and H belonging to insecticide class of Organochlorines (OC) and substance K to Phenyl Pyrazoles. First two mentioned substances were given as LD and HD respectively at 5 or 25 mg/kg bw/d by gavage and 1000 or 3000 ppm in the diet. The concentration of Vinclozolin in the diet refers to doses of 80 and 240 mg/kg bw/d (250 g body weight and 20 g food intake per day) respectively.

Aroclor 1254 exhibited a similar pattern to the previously mentioned substances that cause direct thyroid toxicity meaning that a dose-dependent effect was detected for analytes T4, T3, and rT3 (Figure 34 and Figure 35). For most of these analytes the decline in concentration was already profound after 7 days in the HD group since the mean concentrations at the three sampling days were consistent. The reduction of total TH concentrations ranged from 42-96 % in males and from 51-88 % in females at day 28 whereas overall total T3 concentrations were affected to a lesser extent than total T4 and rT3 concentrations. Total T3 concentrations in both sexes were declined by 42-52 % in the HD groups compared to total T4 and rT3 concentrations which were lowered by 85-96 %.

Vinclozolin overall caused a decrement in TH concentrations, however, to a lesser extent than Aroclor 1254. LD and HD groups were statistically decreased in total T4 concentrations in both sexes whereas T3 in LD and HD groups of only females at day 14 was significant altered (see Figure 36). Mostly total rT3 concentrations were significantly decreased in HD animals, but not consistently at all timepoints as shown in Figure 37.

In conclusion, reduced TH concentrations in rat plasma were established after exposure to liver enzyme inducers.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

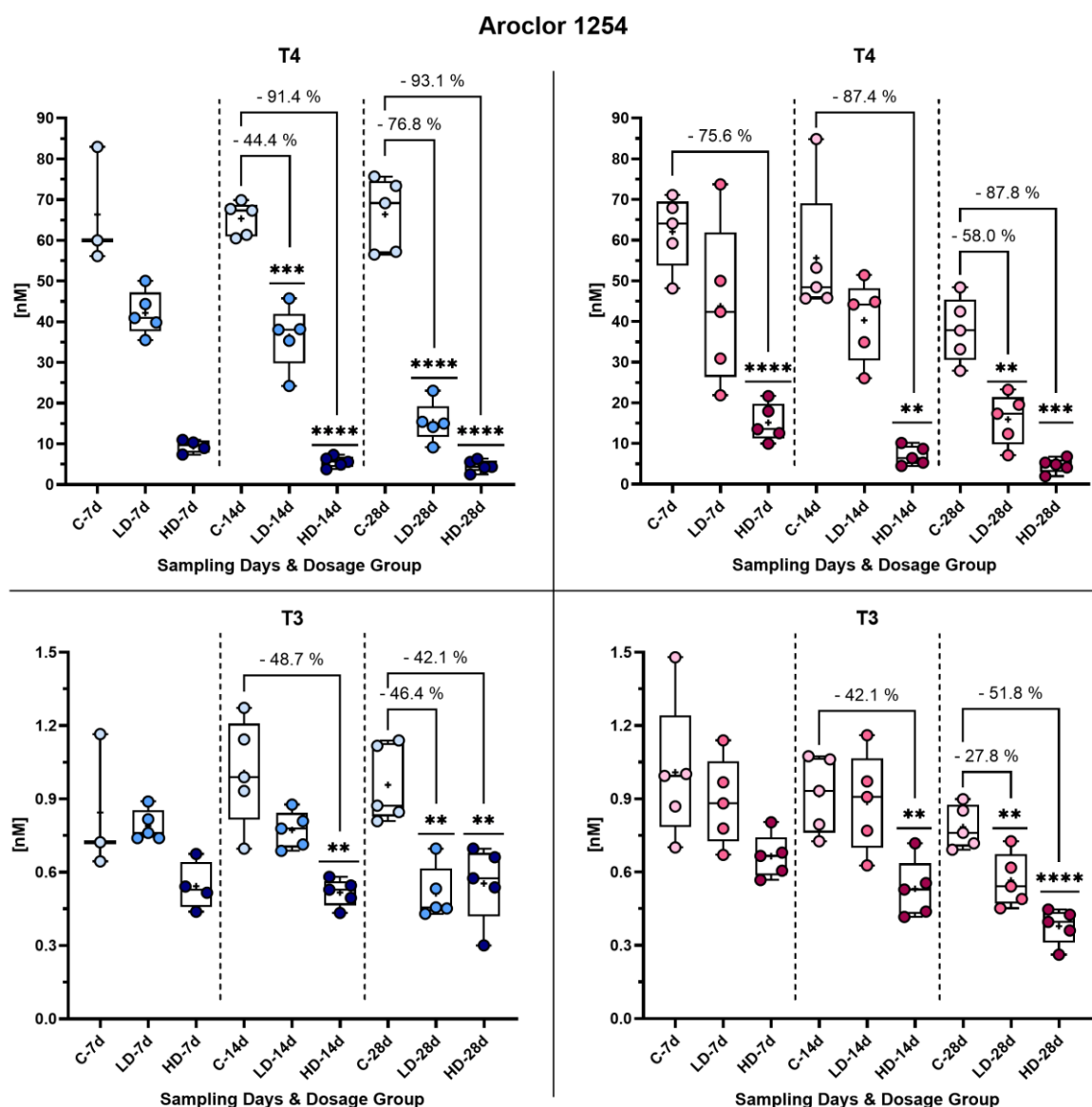


Figure 34. Total T4 and T3 concentrations at three sampling days from control and Aroclor 1254-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

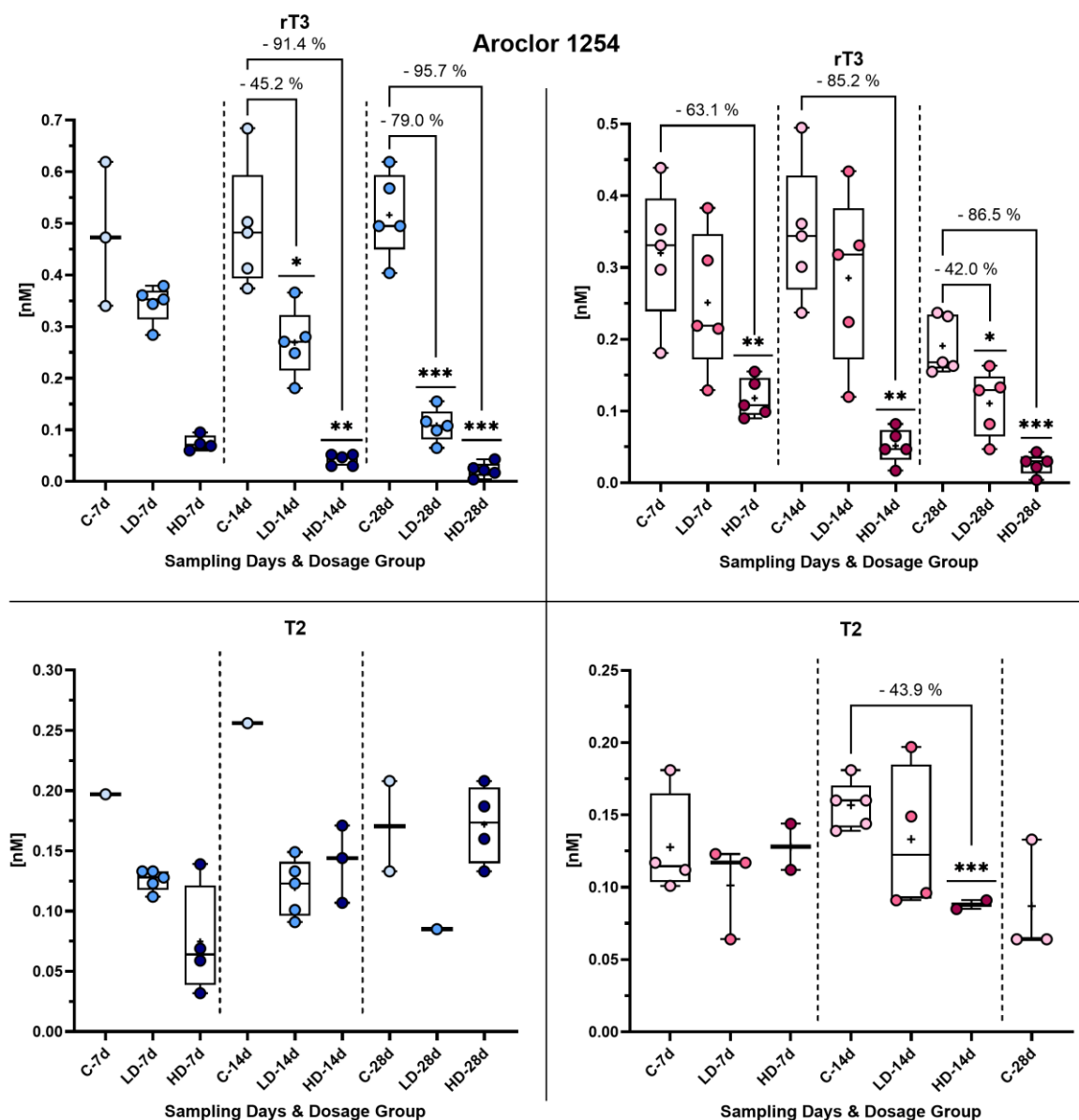


Figure 35. Total rT3 and T2 concentrations at three sampling days from control and Aroclor 1254-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T2 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

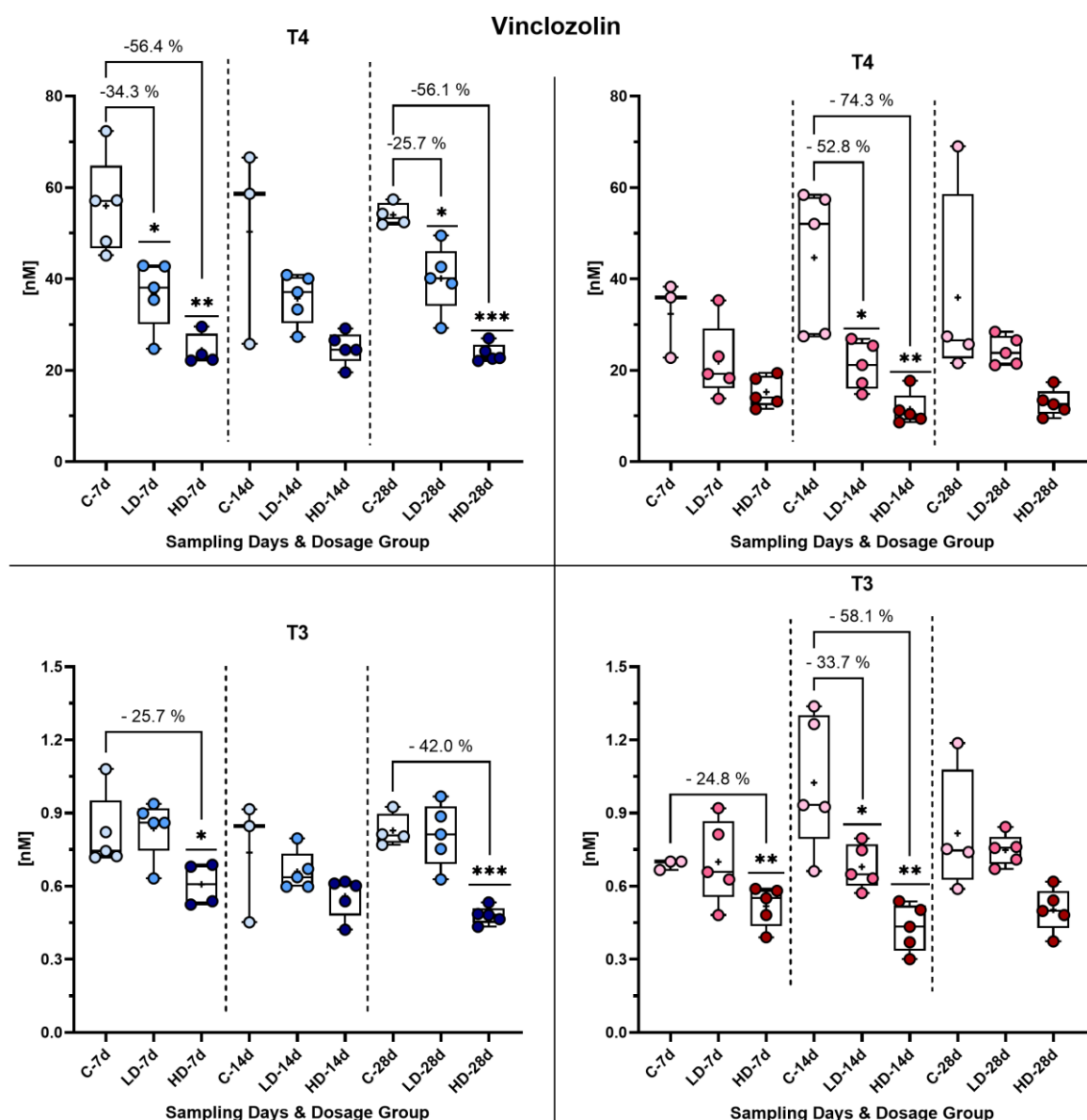


Figure 36. Total T4 and T3 concentrations at three sampling days from control and Vinclozolin-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with **T4** and on the bottom **T3** whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

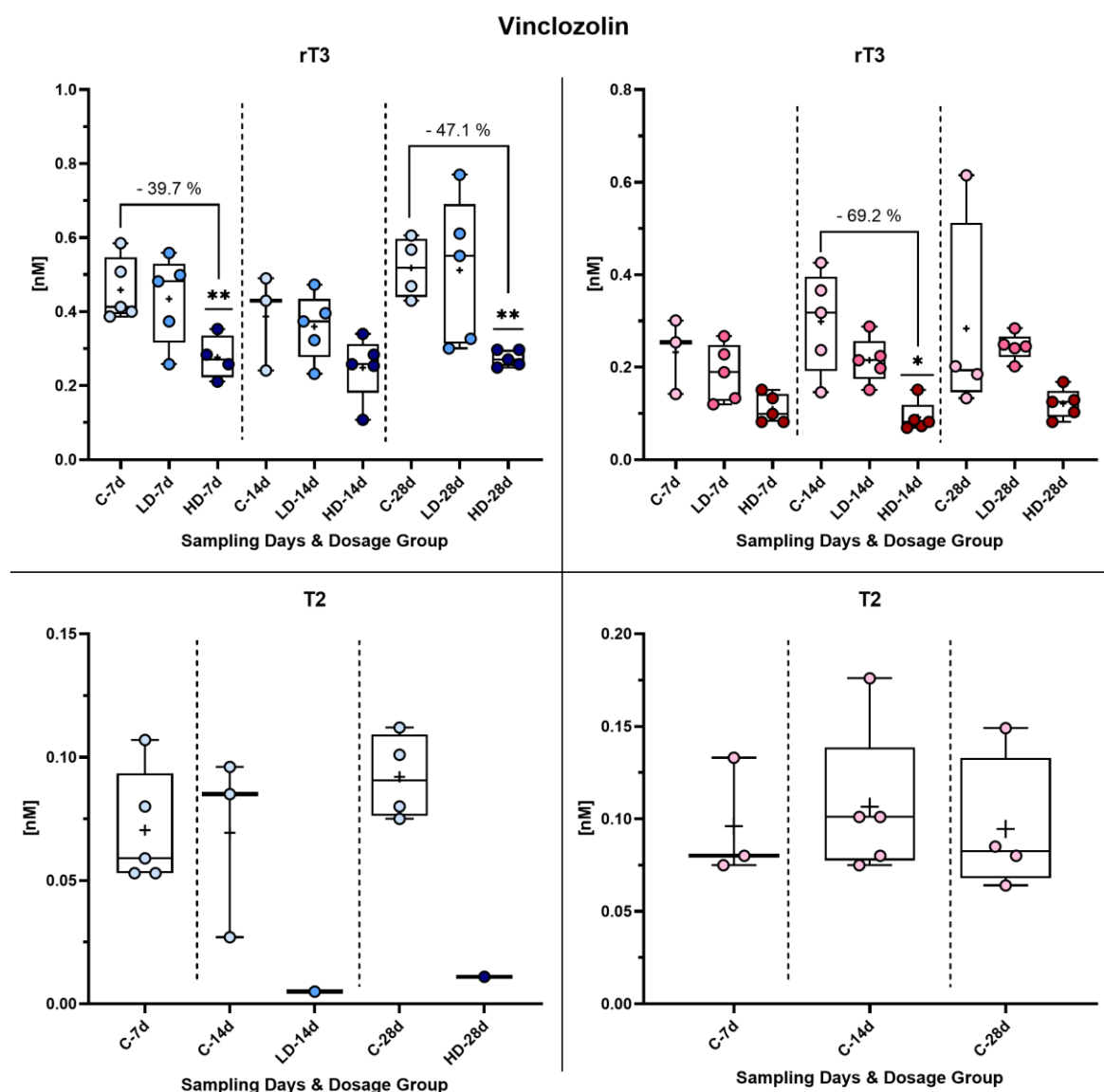


Figure 37. Total rT3 and T2 concentrations at three sampling days from control and Vinclozolin-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with **rT3** and on the bottom **T2** whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

Organochlorines (OC) inhibit the Gamma-aminobutyric Acid (GABA)-gated chloride channel and are further suspected to interact with the TH system by e.g. inducing liver enzymes and thus increase TH clearance or to interact with plasma TH-binding proteins⁷⁵. Substances G and H were administered for 7 days whereas substance G was administered further in a second study for up to 28 days. The first study administered either of the compounds by gavage to rats for a total of 7 days. Substance H was tested in a dose of 5 mg/kg bw/d and substance G of 25 mg/kg bw/d. The second study of substance G included plasma sampling on days 7,14 and 28 in all groups therefore the respective adult rat brain samples were obtained on day 28. TH results of rat plasma are portrayed in Figure 38 and Figure 39 and of rat brain in Figure 40. Substance G exposure after 28 days reduced T4, T3, and T2 concentrations in males and females indicating significance for most of the sampling days. Only two HD samples of male rats on day 28 were analyzed because the remaining replicates did not yield enough volume. Analyte rT3 remained unchanged but showed in the first study a reduction in males after exposure to substance G. T3 and T2 concentrations were unaffected by both compounds after 7-day exposure. On contrary, T4 concentrations in males were significantly lowered for both compounds, but higher in females exposed to substance G. Rat brain TH analysis of the respective adult animals in both studies were overall consistent with the control concentration except for significantly increased T4 and T3 concentrations in female rats administered substance H for 7 days (Figure 40). An alteration by substance G administration in rat brain was detected after 28 days for T4 and rT3 in both sexes, but only in males for T3.

As categorized to affect TH concentrations via an indirect MOAs, the blinded OCs did affect TH concentrations and caused decreased concentrations in rat plasma and brain.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

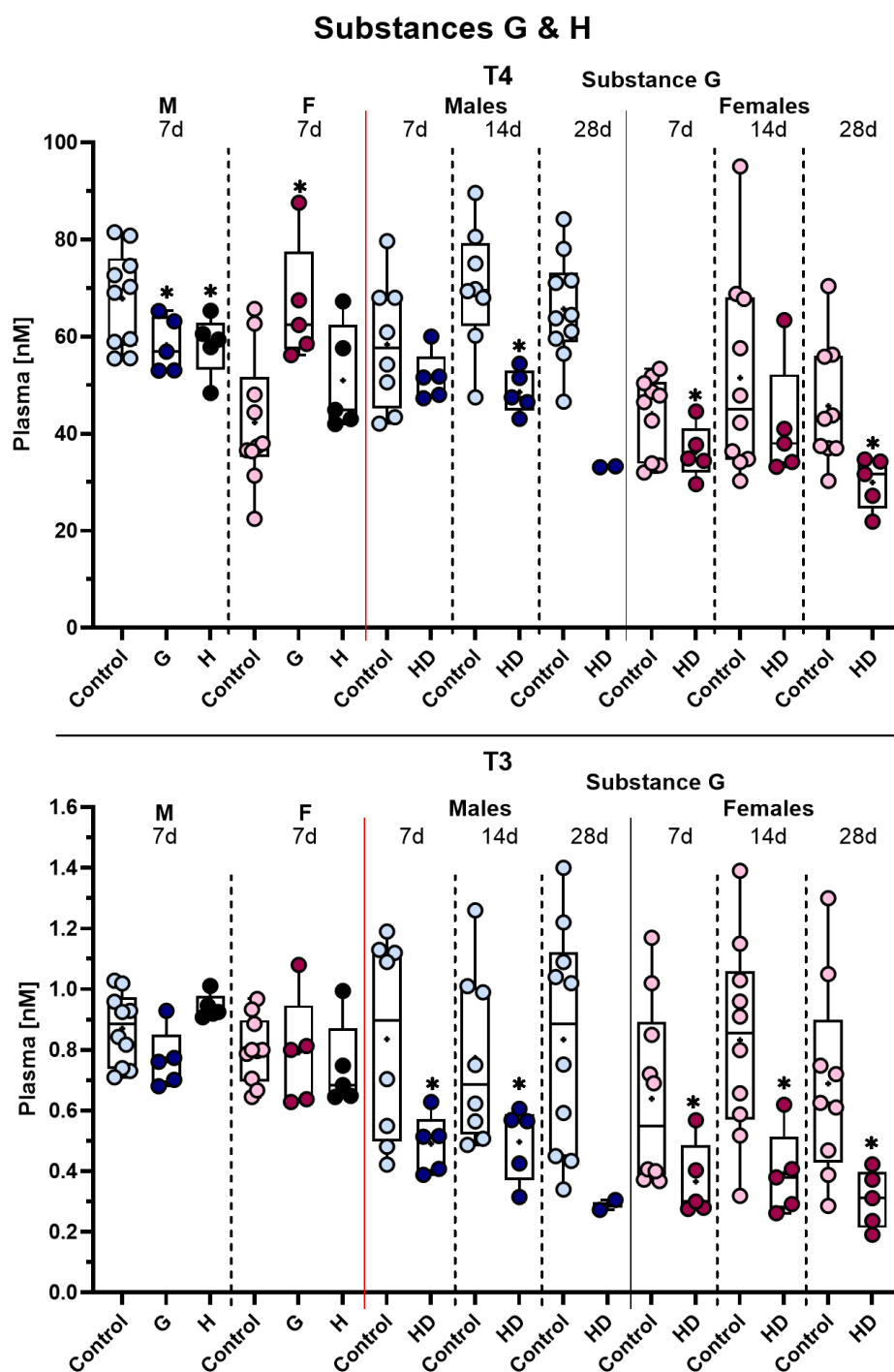


Figure 38. Results from T4 and T3 analysis in rat plasma after exposure to substances G and H.

Rat plasma samples were received from two studies. The first one exposed the rats to either **substance G** or **H** for 7 days and the second one to **substance G** for a total of 28 days, but additionally after day 7 and 14 plasma samples were taken. A separation between the studies is shown by the first vertical red line. The second red line separates males and females results within the second study. Results obtained from either males or females are shown above the box plots and further highlighted by the color of the dots (blue for males and red for females). Concentration is plotted on the y-axis as [nM] and on the x-axis control and substances of the first study as well as control or HD of the second study are listed. In the first study the black dots refer to **substance H** administration and the colored ones to **G**. The graph of **T4** is ordered on top and below of **T3**. The asterisks refer to statistical significance: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

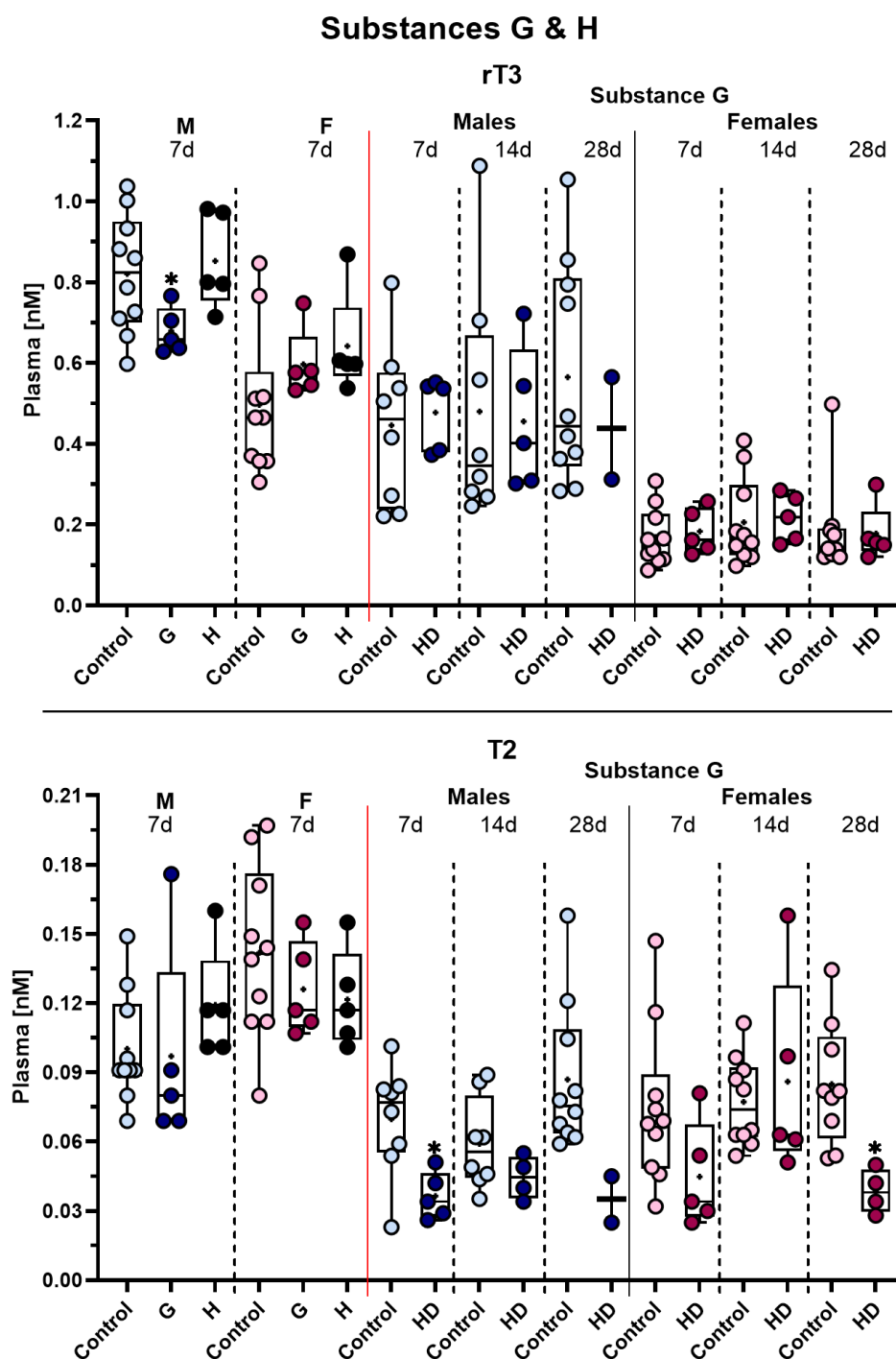


Figure 39. Results from rT3 and T2 analysis in rat plasma after exposure to substances G and H.

Rat plasma samples were received from two studies. The first one exposed the rats to either **substance G** or **H** for 7 days and the second one to **substance G** for a total of 28 days, but additionally after day 7 and 14 plasma samples were taken. A separation between the studies is shown by the first vertical red line. The second red line separates males and females results within the second study. Results obtained from either males or females are shown above the box plots and further highlighted by the color of the dots (blue for males and red for females). Concentration is plotted on the y-axis as [nM] and on the x-axis control and substances of the first study as well as control or HD of the second study are listed. In the first study the black dots refer to **substance H** administration and the colored ones to **G**. The graph of **rT3** is ordered on top and below of **T2**. The asterisks refer to statistical significance: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

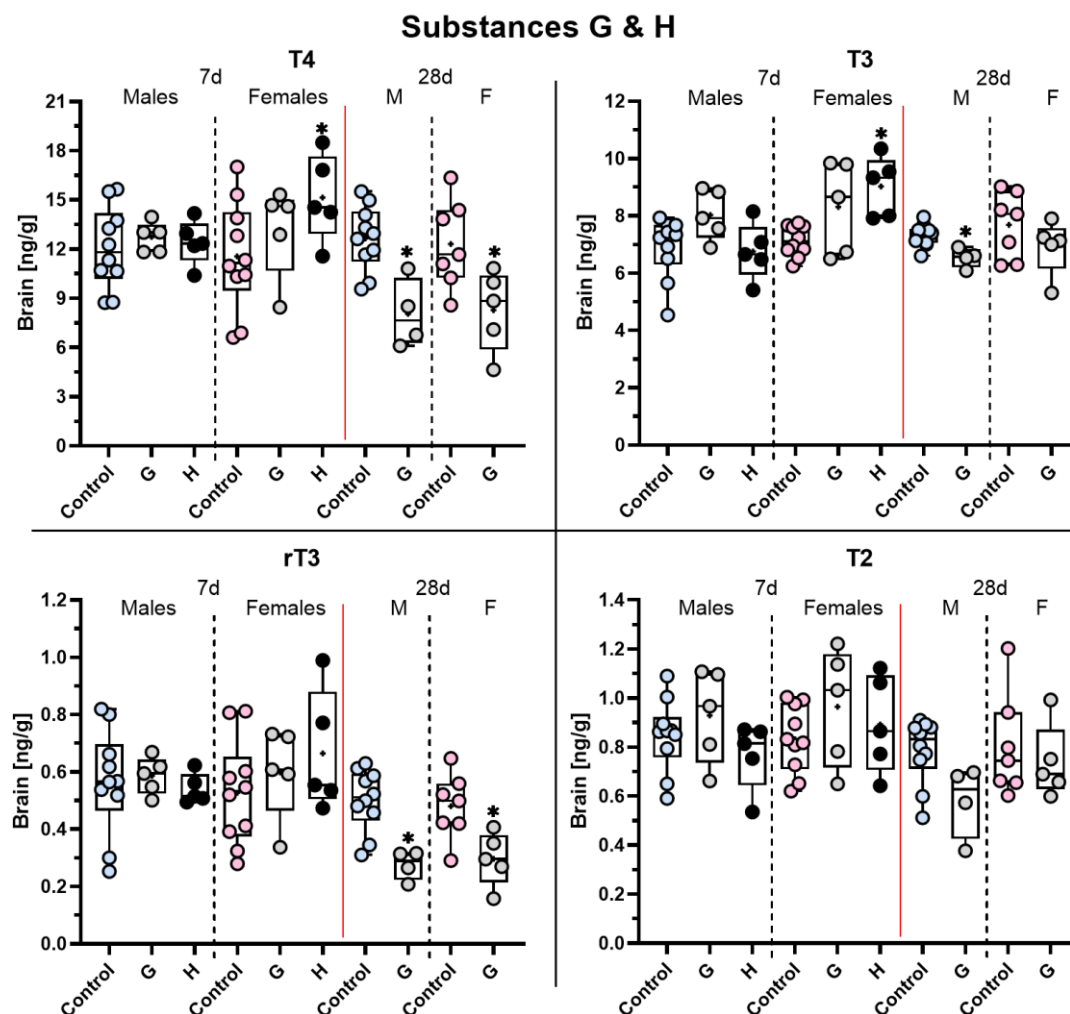


Figure 40. Effect of substances G and H on TH concentrations in adult rat brain.

The box plot graphs are plotted in an order from T4, T3, rT3 to T2. The vertical red solid line in each graph divides the data sets obtained from two different studies and males and females are separated by a dotted black line. Control and substance groups are mentioned on the x-axis whereas the color of the data points from the control groups indicate the results obtained from either males (blue) or females (red) which is also named above the box plots. Substances are differentiated by the dots in color light grey for **substance G** and black for **substance H**. Statistical significance is shown by the asterisks: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

The last analyzed substance K was administered to adult rats through the diet containing 400 ppm for 7 days which equals a dose of 32 mg/kg bw/d. The here presented TH analysis in the given rat plasma and brain are shown in Figure 41 and Figure 42. Figure 41 represented yielded data in rat plasma pairwise for control and dosed groups as well as males and females. Detected T4, T3 and rT3 concentrations were significant reduced compared to the control group in male rats. A significant reduction in female rats was only detected for analyte T3. On contrary, T2 concentrations significant increased after exposure to substance K in males and females. Results from TH analysis in the respective brains demonstrated a significant reduction in males for T4 and in males and females for T2 whereas T3 concentration in female rats significant increased. The analyte rT3 was unchanged in brain after exposure to substance K.

As concluded for OCs, an indirect TH-related MOA is suspected, the blinded Phenyl Pyrazole also caused decreased TH concentrations in rat plasma which was not mirrored in TH concentrations in rat brain.

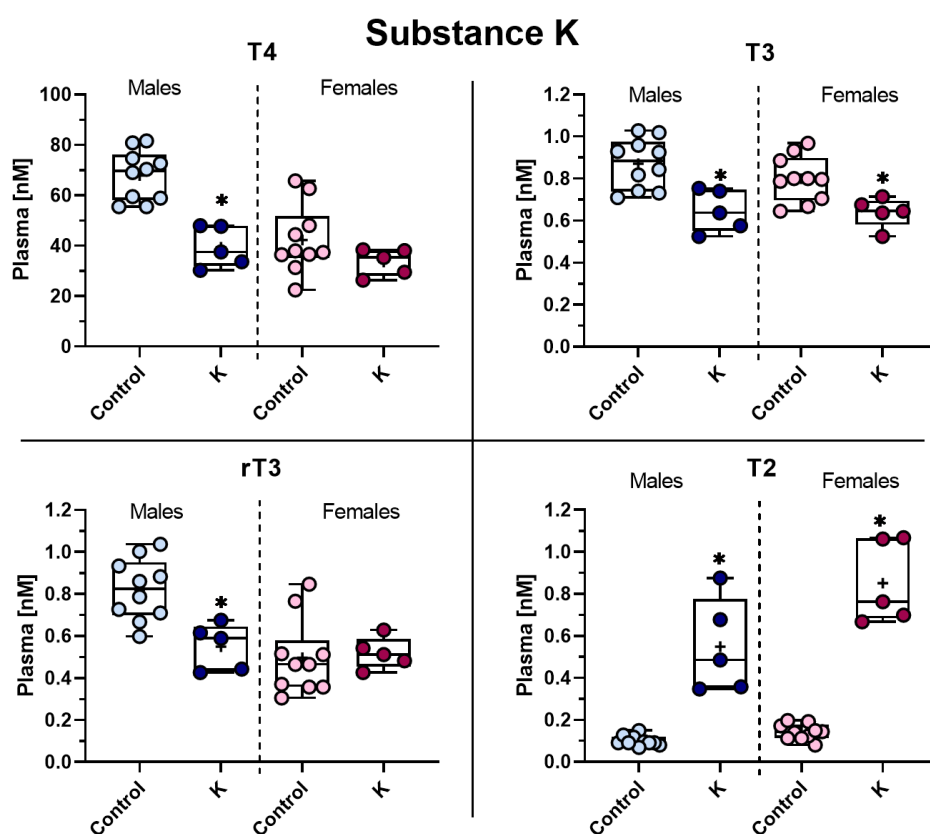


Figure 41. TH concentrations in rat plasma exposed to HD of substance K after 7 days.

Analytes T4 to T2 are plotted in subsequent order from left to right showing pairwise the TH results for males and females compared to the given control which is indicated on the x-axis. Blue color refers to male and red color to female rats. Statistical significance is shown by the asterisks: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

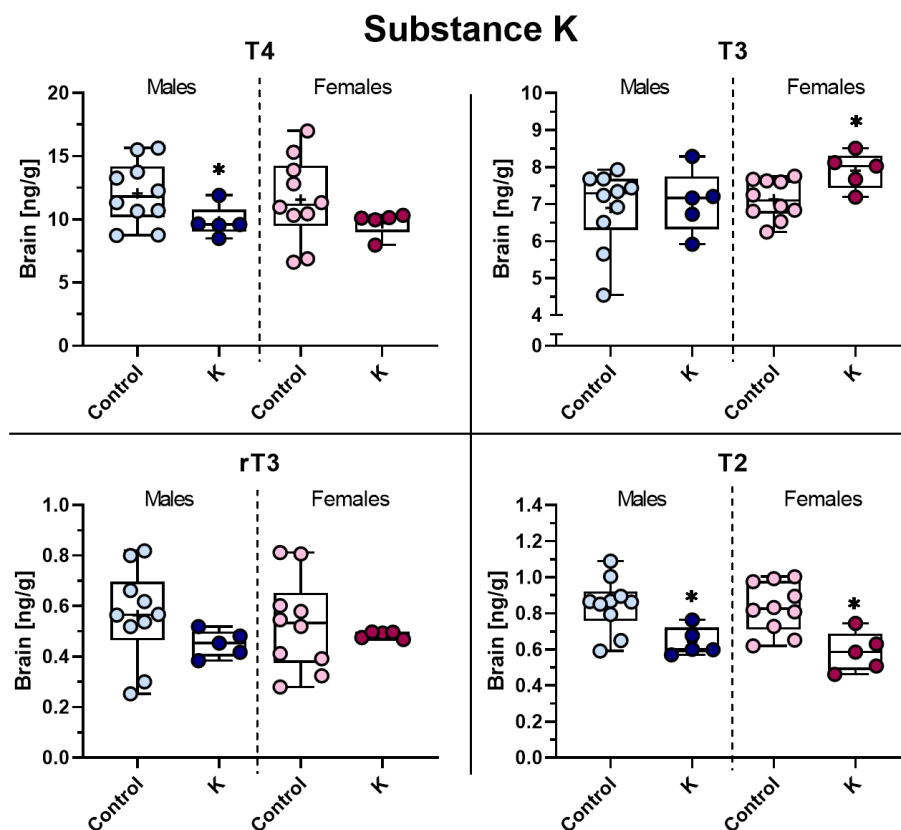


Figure 42. TH concentrations in adult rat brain exposed to HD of substance K after 7 days.

Analytes T4 to T2 are plotted in subsequent order from left to right showing pairwise the TH results for males and females compared to the given control which is indicated on the x-axis. Blue color refers to male and red color to female rats. Statistical significance is shown by the asterisks: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.2.2 Effect on Total TH Concentrations by Plasma TH-Binding Protein Substances

The competitive binding of xenobiotics to the plasma TH-binding proteins namely TBG, TTR or albumin causes a decline in total TH concentration in plasma. Exemplary xenobiotics are TBBPA or Perfluorohexanesulphonic Acid (PFHxS) which effect to alter TH in plasma was estimated and shown in the following figures. TBBPA was administered by gavage in doses of either 300 or 1000 mg/kg bw/d and PFHxS of either 6 or 20 mg/kg bw/d.

TBBPA caused decline of total T4, and T3 concentrations in both sexes as depicted in Figure 43 whereas the effect was more persistent in males showing reduced concentrations at all sampling days. At all sampling days the reduced T4 concentrations in males were similar ranging from 54-64 % in the LD group and from 64-74 % in the HD group as well as for T3 from 31-43 % in the LD group and from 38-53 % in the HD group. In females T4 concentrations were similar at day 7 and 14 in both dosage groups, but after 28 days the reduction of T4 concentration increased from around 33 to 62 % in the LD group and from 44 % to 74 % in HD group compared to the control group. Total T3 concentrations in females were only significantly altered in both groups at day 14. Like the effect seen in T4 in males, total rT3 concentrations were affected in a comparable pattern illustrated in Figure 44. At every sampling day male rats from the LD groups yielded lowered rT3 concentrations consistently by around 57-58 % compared to the control group and by around 63-74 % in the HD groups. Notable in male rats was further that the mentioned reduction was the strongest on day 7. Moreover, analyte T2 was unaffected in males but overall, significantly increased in females compared to T2 concentration of the control group (Figure 44).

PFHxS affected especially total T4 concentration in males stronger than TBBPA. Total T4 concentrations in LD and HD groups as well as on every sampling day were comparable lowered by 85-90 % compared to the control. This effect was observed as well for total T3 concentrations which were reduced by 58-65 %. In females total T4 was less affected than after 28-day exposure of TBBPA but reduction was consistently by around 50-58 % in the HD groups. Total T3 concentrations in females were significantly altered by around 20-28 % in the HD group after 14 days as well as in the LD group after 28 days. These observations are demonstrated in Figure 45. Similar to T4, total rT3 was strongly reduced in males at all days by around 82-86 % and in females by 46-73 % as shown in Figure 46. Detected T2 concentrations were mainly unchanged in both sexes except for a decline in males at day 14 which was negligible at day 28. Concentrations of T1 was detected in only females and were unaffected except at day 7 after PFHxS exposure. As already stated for TBBPA, the estimated reduction for analytes T4, T3, and rT3 weakened from day 7 to 28.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

In conclusion, TBBPA and PFHxS reduced TH concentrations in rat plasma, but interestingly T3 was less strongly affected in females than in males.

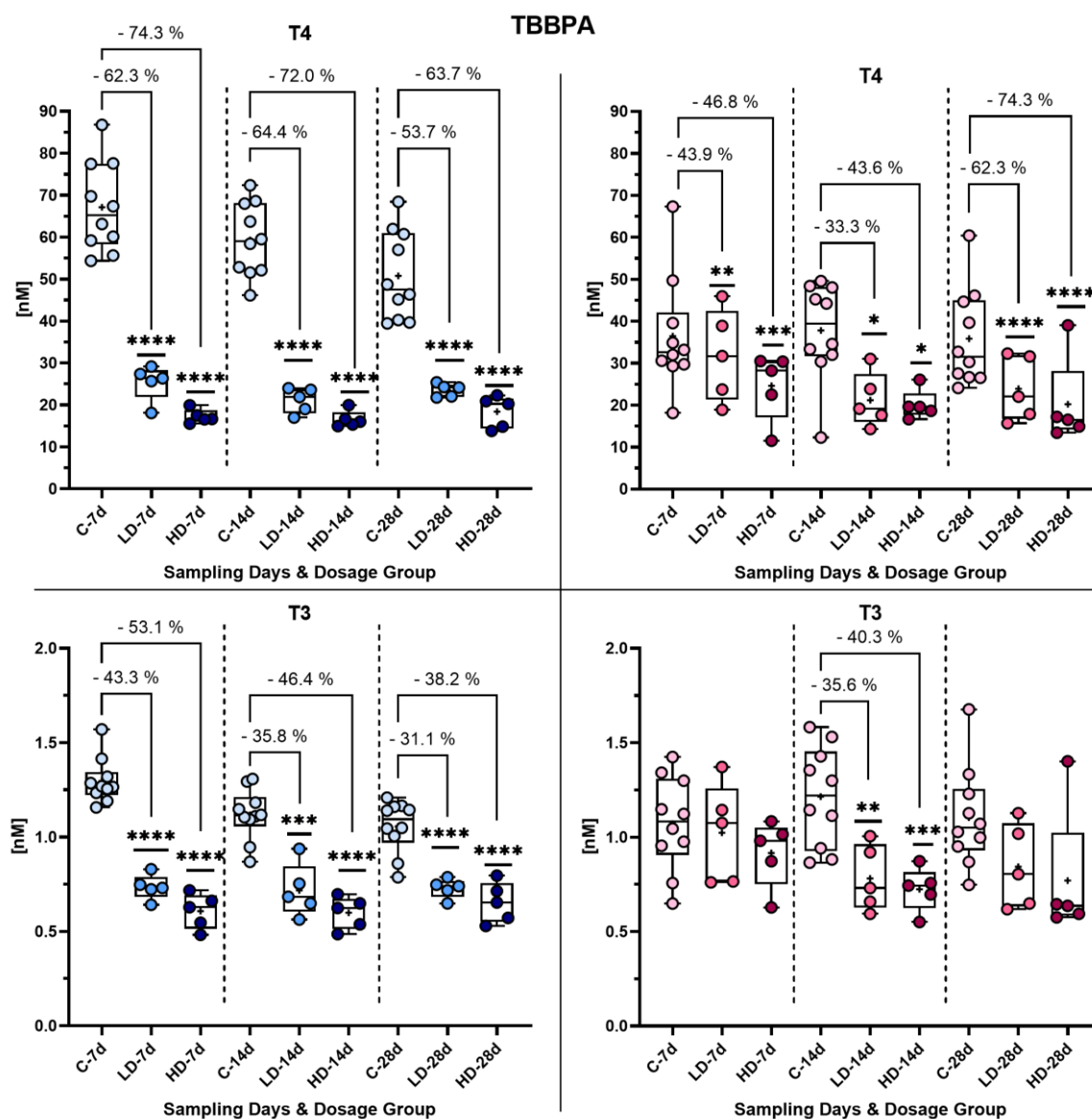


Figure 43. Total T4 and T3 concentrations at three sampling days from control and TBBPA-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

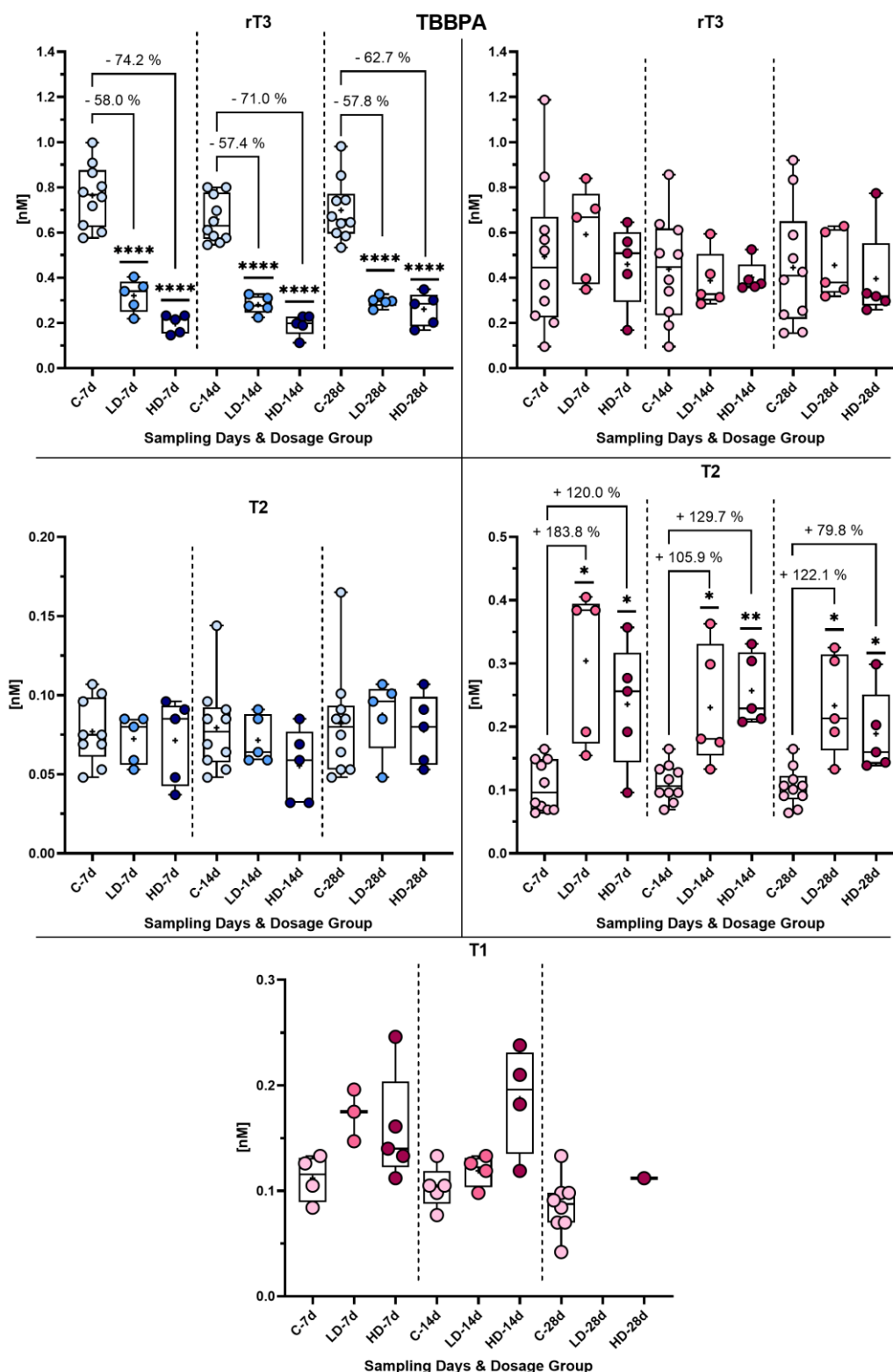


Figure 44. Total rT3, T2 and T1 concentrations at three sampling days from control and TBBPA-exposed rats.

Results for male and female rats are listed pairwise for each analyte starting from top with rT3 and on the bottom T1 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

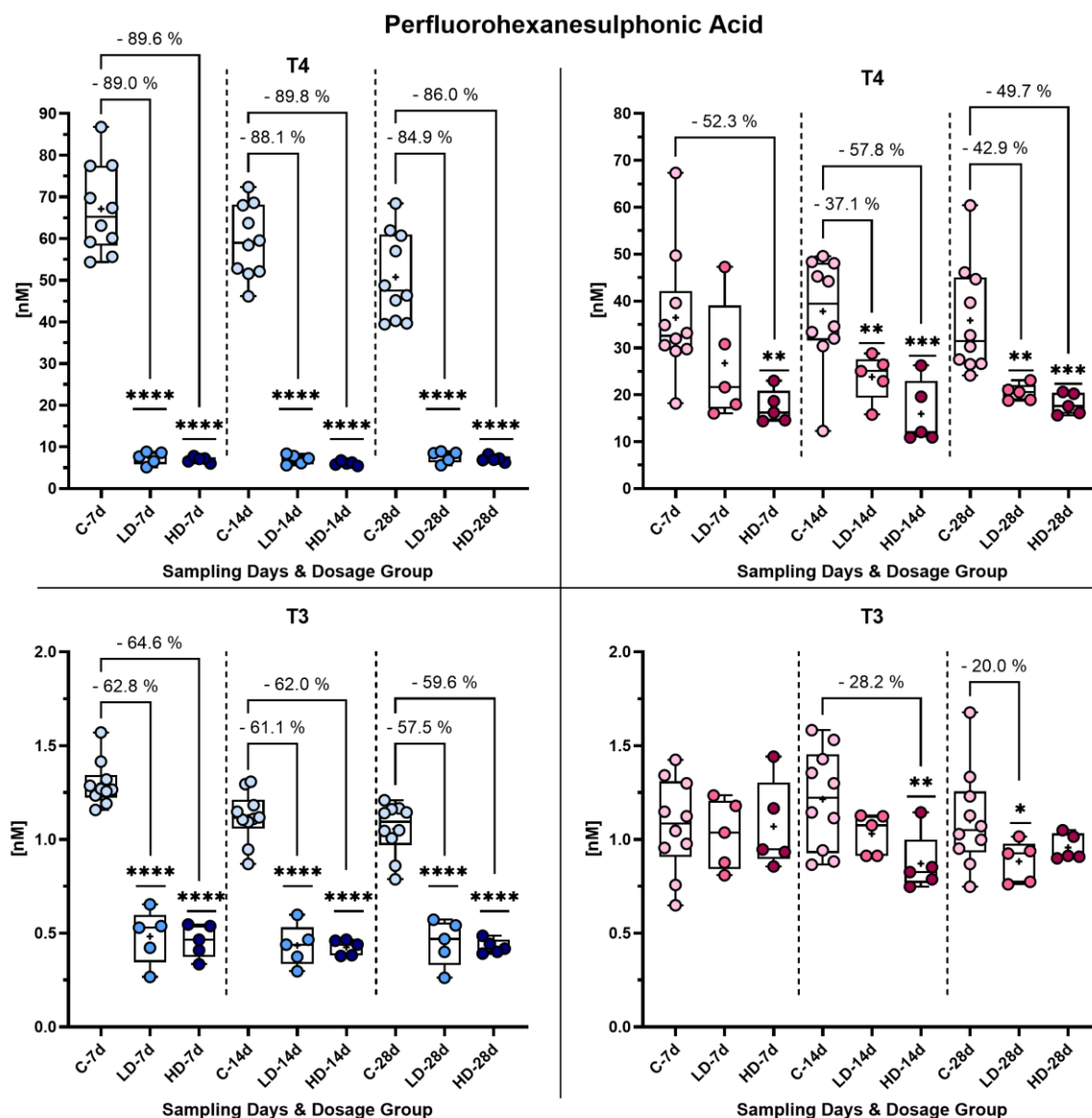
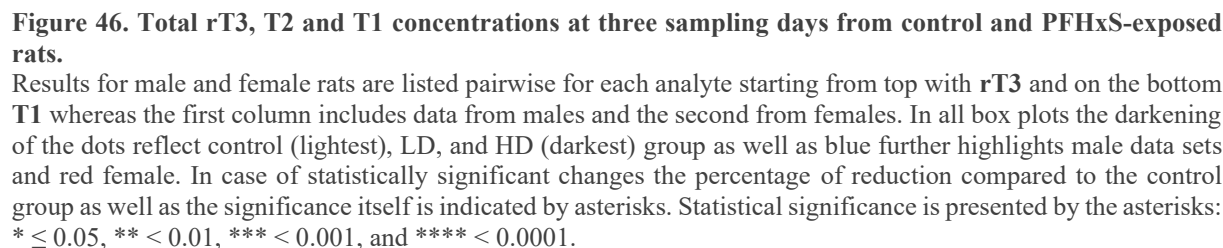


Figure 45. Total T4 and T3 concentrations at three sampling days from control and PFHxS-exposed rats. Results for male and female rats are listed pairwise for each analyte starting from top with T4 and on the bottom T3 whereas the first column includes data from males and the second from females. In all box plots the darkening of the dots reflect control (lightest), LD, and HD (darkest) group as well as blue further highlights male data sets and red female. In case of statistically significant changes the percentage of reduction compared to the control group as well as the significance itself is indicated by asterisks. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

Perfluorohexanesulphonic Acid



5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.3 Miscellaneous TH-Related MOAs

5.7.3.1 TH Data Detected in Rat Plasma and Brain after Exposure to Pyrethroids

Pyrethroids are a class of synthetic pesticides which were derived from the natural occurring pyrethrin in Chrysanthemum flowers which paralyze the insecticide by preventing the closure of voltage-gated Sodium channels¹⁴⁰. From this class three representative substances were tested namely substances A, B, and C. Substance A was examined in a 7-day exposure study and given through the diet in a concentration of 150 ppm. Substance B was administered through the diet in concentrations of 400 ppm (LD) or 800 ppm (HD) and substance C orally by gavage at doses of either 50 mg/kg bw/d (LD) or 150 mg/kg bw/d (HD). In a separate 7-day study the animals received a dose of 150 mg/kg bw/d substance C by gavage. The concentrations of substance A and B in the diet was calculated to the respective doses considering 250 g body weight and a food consumption of 20 g per day. This yielded doses of 12 mg/kg bw/d for substance A and of 32 and 64 mg/kg bw/d for substance B respectively. Substance A did not cause significant alterations of none of the analytes nor matrix and the results are presented in Figure 47. Figure 48 represented the total TH concentrations in plasma after exposure to substance B. T3, rT3, and T2 concentrations were for the most part increased after exposure to LD and HD whereas T4 concentration on contrary decreased. Figure 49 and Figure 50 comprising total TH concentrations in plasma after substance C exposure at different sampling days as well as the data from the separate 7-day study was included shown first in the figure. The HD in female rats after 28 days only yielded three data points (n=3). T4 concentrations were dose dependently affected causing a statistically significant decrease of its concentration in the HD group at every sampling day in males. Similar as for substance B, T3, rT3, and T2 yielded rather a tendency of increase which was more noticeable in the LD than HD group and they were statistically significantly altered at a few of the days examined. On the last sampling day which was for one study at day 7 and the other day 28, adult rat brains were sampled besides plasma. The analysis of TH concentrations in these sampled adult whole rat brains from three different studies are illustrated in Figure 51 and separation of different studies data are outlined by a red line. Bright blue and bright red colored dots symbolize male and female control animals whereas the dark grey dots represent substance B and black dots substance C exposed rats. TH analysis of whole rat brain was overall not statistically

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

significantly altered except for T4 and rT3 in female rats after 28-day exposure to substance B (increase) and rT3 after exposure to substance C for 28 days (decrease).

Conclusively, the blinded pyrethroids caused a mixed TH alterations pattern such as substance A did not cause any alteration, but substances B and C decreased T4 but increased T3 concentrations in plasma. Overall, TH alterations in rat brain were mostly non-significant.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

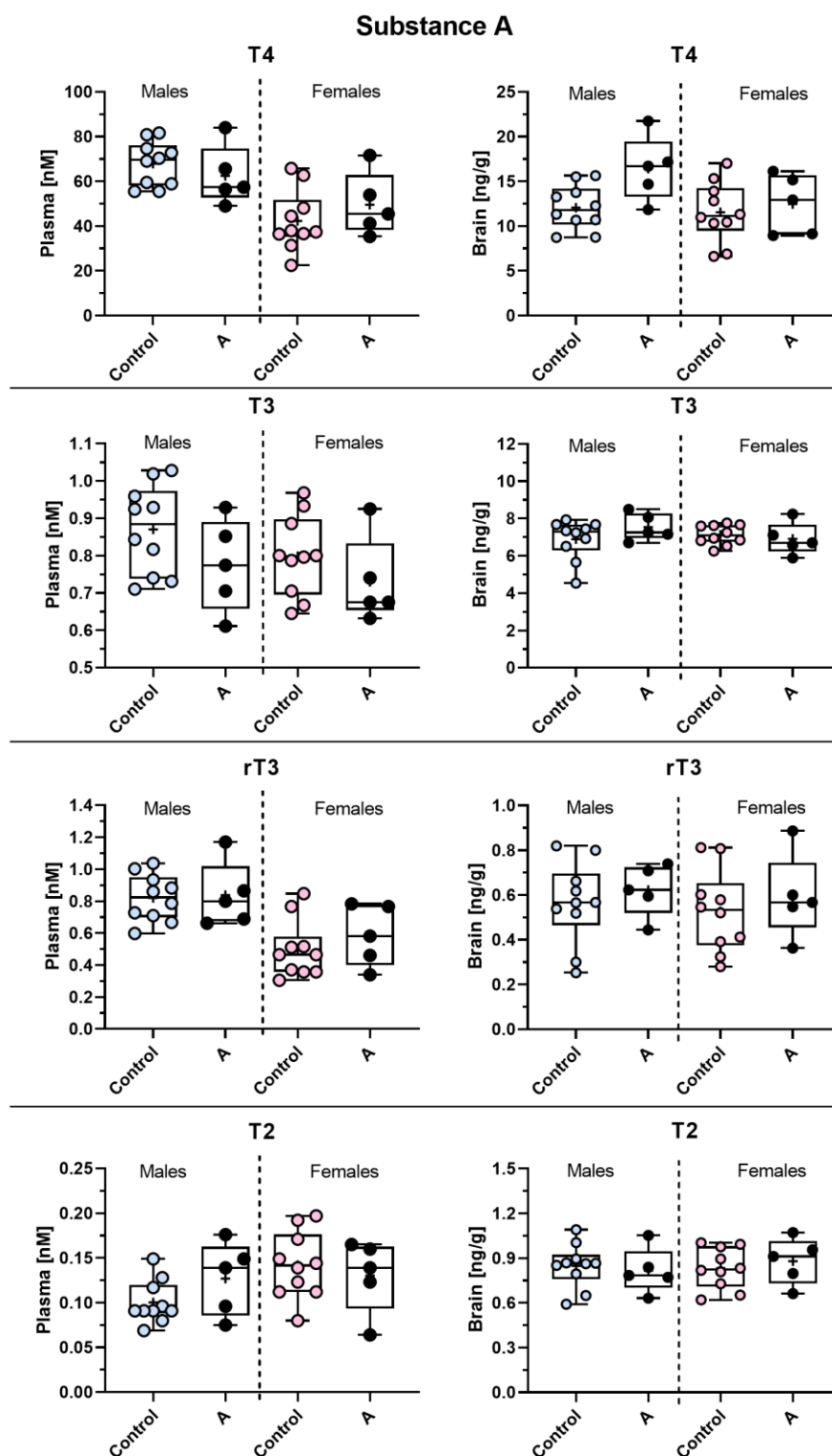


Figure 47. TH concentrations in brain and plasma of adult rats exposed to substance A for 7 days.

In each column TH analytes are ranked in subsequent order starting with T4 and ending with T2. Plasma results are plotted in the first column on the left and the respective brain TH results in the same order in the column to the right. Each graph shows control and **substance A** group on the x-axis contrasted to the concentration [nM] on the y-axis. The dotted line separates the data set from males and females which is further written above the box plots. The dots of the control groups are colored in blue (males) or red (females) and substance A data points are colored in black. No statistical significance was detected.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

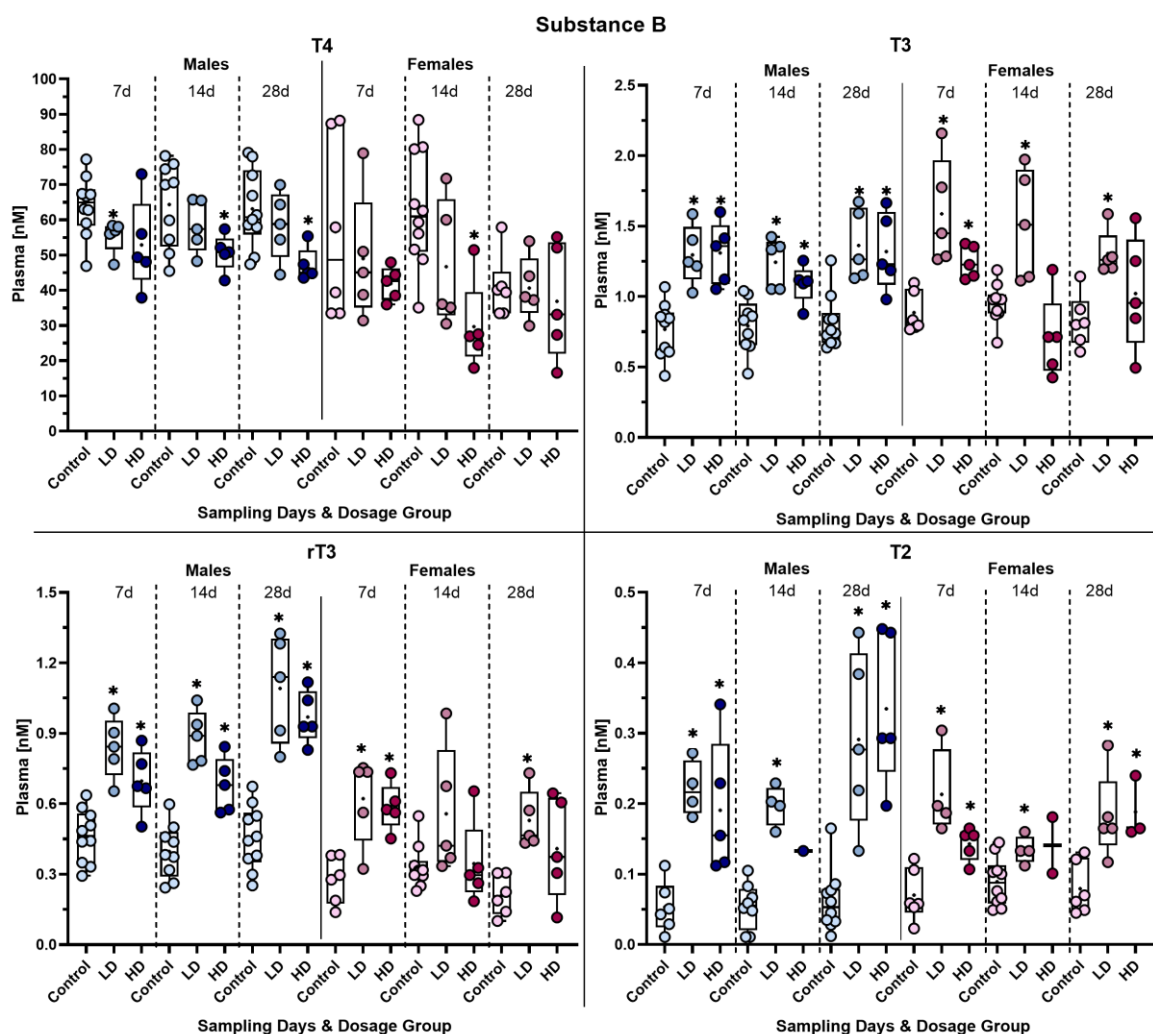


Figure 48. Analysis of rat plasma regarding their TH concentration after exposure to substance B.

Control, LD and HD groups are plotted on the x-axis in contrast to plasma concentration in [nM] on the y-axis. This rank of the given groups is emphasized by the darkening color of the dots. The color also indicates data sets of males (blue) from females (red). Sampling days (divided by dotted line) and sex are named above the box plots. The top graphs include **T4** and **T3** whereas below **rT3** and **T2** are placed. The asterisks highlight statistical significance: $* \leq 0.05$.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

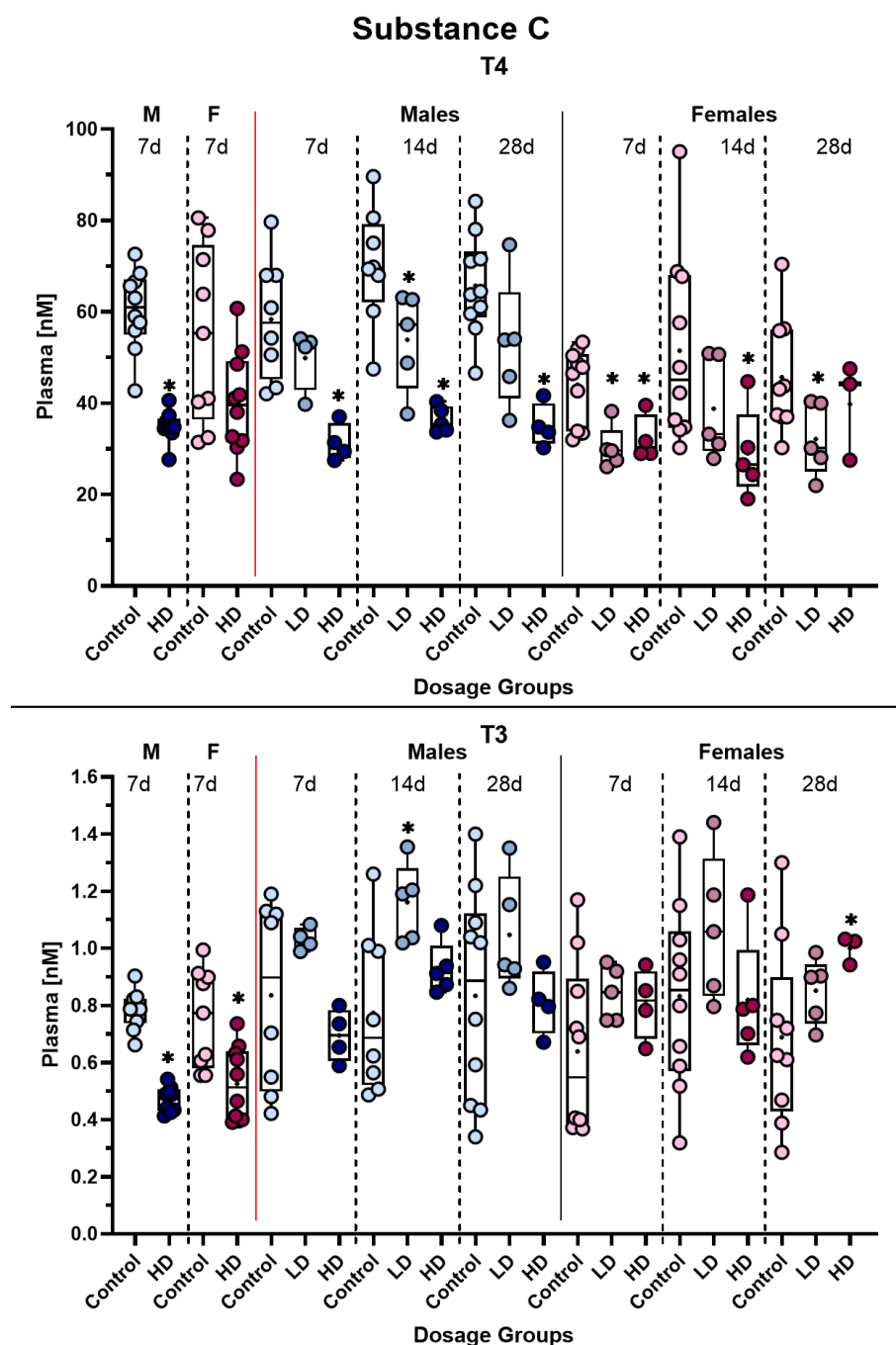


Figure 49. Analysis of rat plasma regarding their T4 and T3 concentration after exposure to substance C. **Substance C** was applied to rats in two separate studies indicated in the graph by the first vertical red line. The first study lasted 7 days and the second in total 28 days, however extra sampling occurred on day 7 and 14. Control, LD and HD groups are plotted on the x-axis in contrast to plasma concentration in [nM] on the y-axis. This rank of the given groups is emphasized by the darkening color of the dots. The color also indicates data sets of males (blue) from females (red). Sampling days (divided by dotted line) and sex are named above the box plots. The top graph depicts results for **T4** and below for **T3**. The asterisks highlight statistical significance: $* \leq 0.05$.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

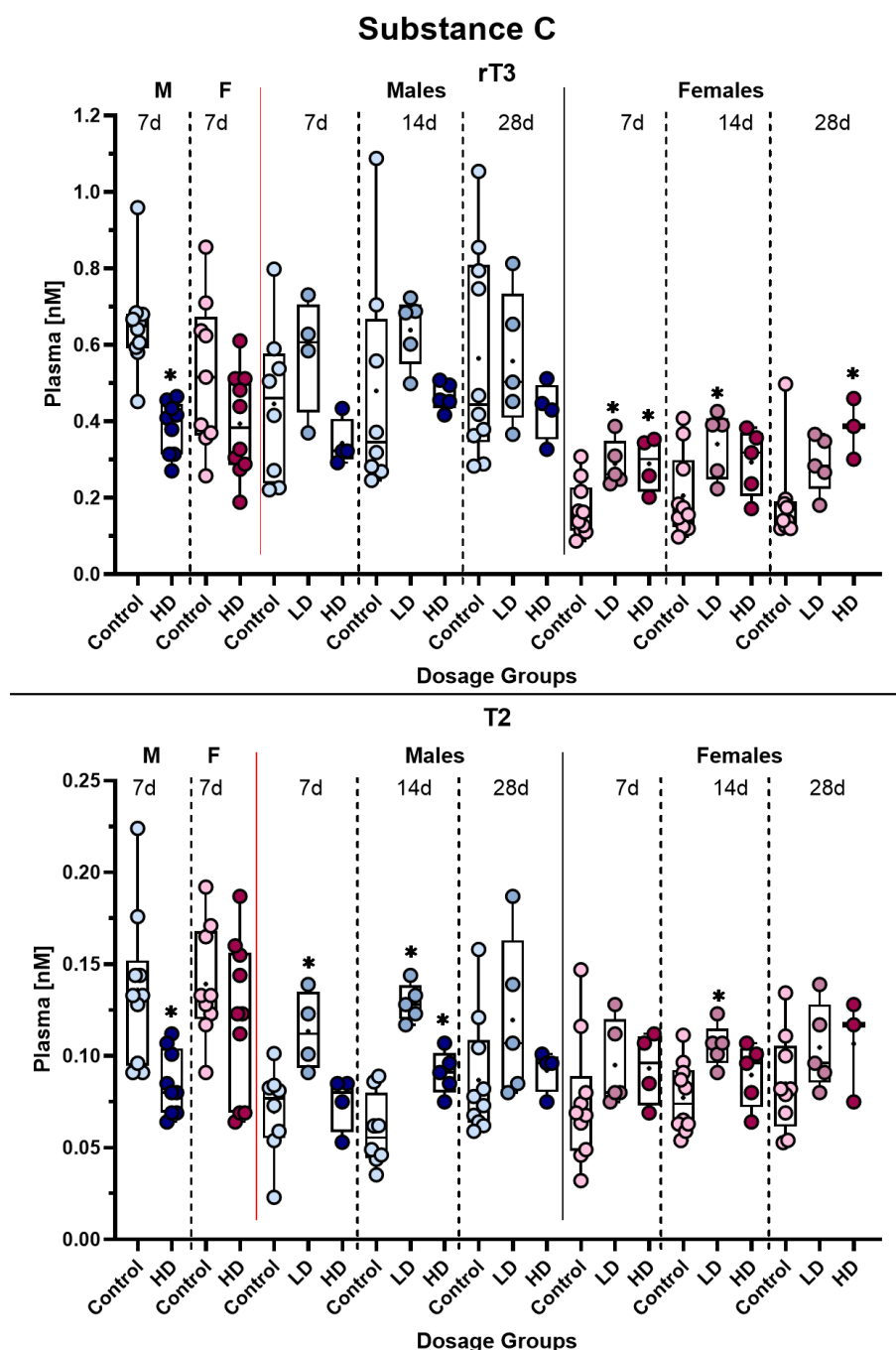


Figure 50. Analysis of rat plasma regarding their rT3 and T2 concentration after exposure to substance C. **Substance C** was applied to rats in two separate studies indicated in the graph by the first vertical red line. The first study lasted 7 days and the second in total 28 days, however extra sampling occurred on day 7 and 14. Control, LD and HD groups are plotted on the x-axis in contrast to plasma concentration in [nM] on the y-axis. This rank of the given groups is emphasized by the darkening color of the dots. The color also indicates data sets of males (blue) from females (red). Sampling days (divided by dotted line) and sex are named above the box plots. The top graph depicts results for rT3 and below for T2. The asterisks highlight statistical significance: $* \leq 0.05$.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

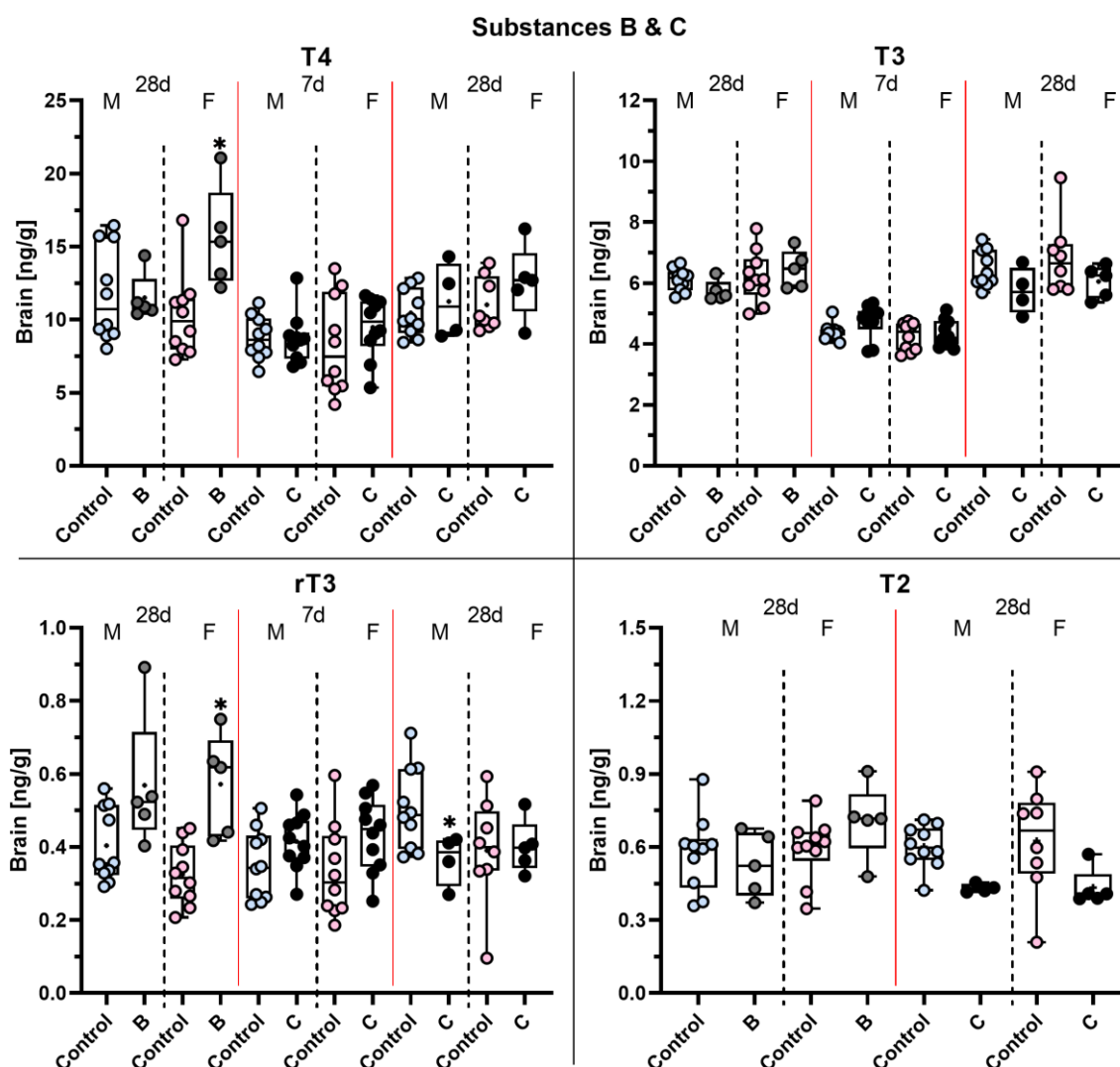


Figure 51. Analysis of adult rat brain regarding their TH concentrations after exposure to substance B and C.

THs were analyzed after an exposure period of 28 days for both compounds whereas **substance C** additionally after 7 days. The sampling days and sex are given above the box plots which are further divided by study through a red line. The dotted black lines separate male from female data sets. Male and female control rats are marked by colored dots in either blue or red respectively whereas the compounds are grey referring to **substance B** and black to **substance C**. T2 data after 7-d exposure to **substance C** were inconclusive. Potential statistically significant differences are indicated by the asterisks highlight statistical significance: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.3.2 TH Changes in Plasma after Exposure to Organotin Compounds

Similar to OC, Phenyl Pyrazoles inhibit GABA-gated chloride channel thereby disrupting the CNS of the insecticides. Organotin pesticides inhibit the mitochondrial adenosine triphosphate (ATP) synthase. Substances I and J were administered once for 7 days and substance I additional for 28 days. Last mentioned exposure further included sampling after day 7 and 14 for plasma analysis as well as LD and HD were available. The HD was equivalent to the administered dose of substance I in the first study for 7 days which was 10 mg/kg bw/d in corn oil by gavage. The LD was dosed at 3 mg/kg bw/d in corn oil by gavage. Substance J was administered in the diet with a concentration of 60 ppm which equaled a dose of 4.8 mg/kg bw/d assuming a body weight of 250 g and a food consumption of 20 g per day. Figure 52 and Figure 53 displayed plasma TH analysis data from both studies showing a significant reduction of T4 after HD exposure of substance I at all sampling days for males and in females after day 7 and 14 in the second study. T3 concentration was significantly reduced after 7 days as seen in the first study in males, but in the second study this was not reproducible due to a larger variation of the given control group. Notably, was the higher standard deviation of the control groups in the second study compared to the first which was true for all analytes. T3, rT3 and T2 concentrations in males from the second study revealed an intermediate significant increase in the LD on all sampling days which was not seen in females. Exposure to substance J for 7 days decreased T4, T3 and rT3 concentrations significantly in males and females whereas T2 concentration increased in males. The corresponding TH analysis in adult rat brain depicted in Figure 54 plotted each analytes graph from left to right starting with T4. Substance I exposure is indicated by light grey data points and substance J by black data points in the box plot graph. After 7-day exposure to substance J, T4 and T2 concentrations in males were significantly decreased whereas T2 was also reduced in females and males when exposed to substance I for 7 days. This was also detected in females for analyte rT3. All analytes demonstrated significantly lowered concentration in both sexes after 28-day exposure to substance I.

The assumption that organotin substances are suspected to further interact via TH-related MOAs was observable because each substance caused reduced TH concentrations in rat plasma. 28-day exposure of substance I further caused reduced TH concentrations in adult rat brain.

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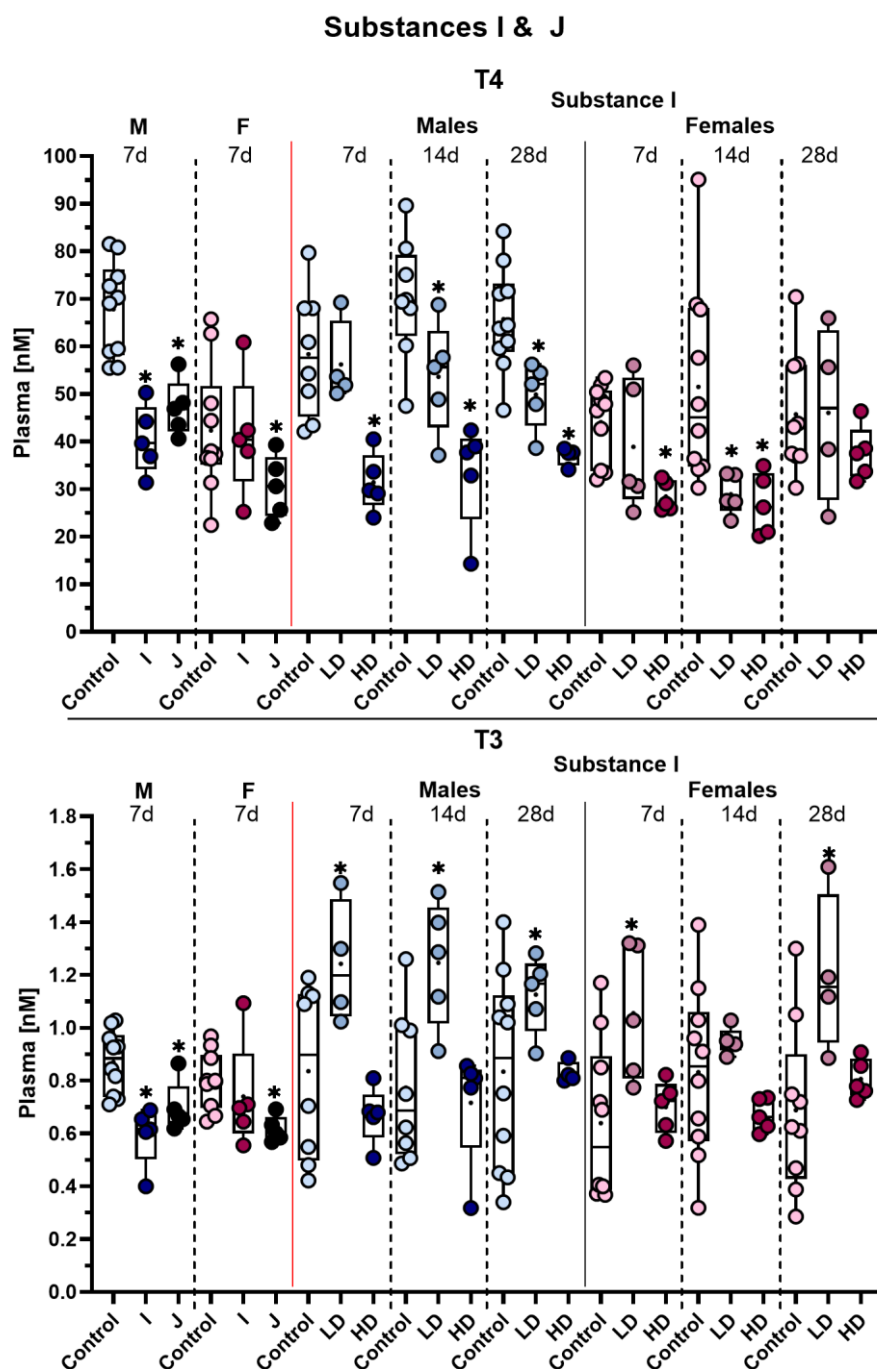


Figure 52. Results from T4 and T3 analysis in rat plasma after exposure to substances I and J.

Rat plasma samples were received from two studies. The first one exposed the rats to either **substance I** or **J** for 7 days and the second one to **substance I** for a total of 28 days, but additionally after day 7 and 14 plasma samples were taken. A separation between the studies is shown by the first vertical red line. Results obtained from either males or females are shown above the box plots and further highlighted by the color of the dots (blue for males and red for females). Concentration is plotted on the y-axis as [nM] and on the x-axis control and substances of the first study as well as control or HD of the second study are listed. In the first study the black dots refer to **substance J** administration and the colored ones to **I**. The graph of **T4** is ordered on top and below of **T3**. The asterisks refer to statistical significance: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

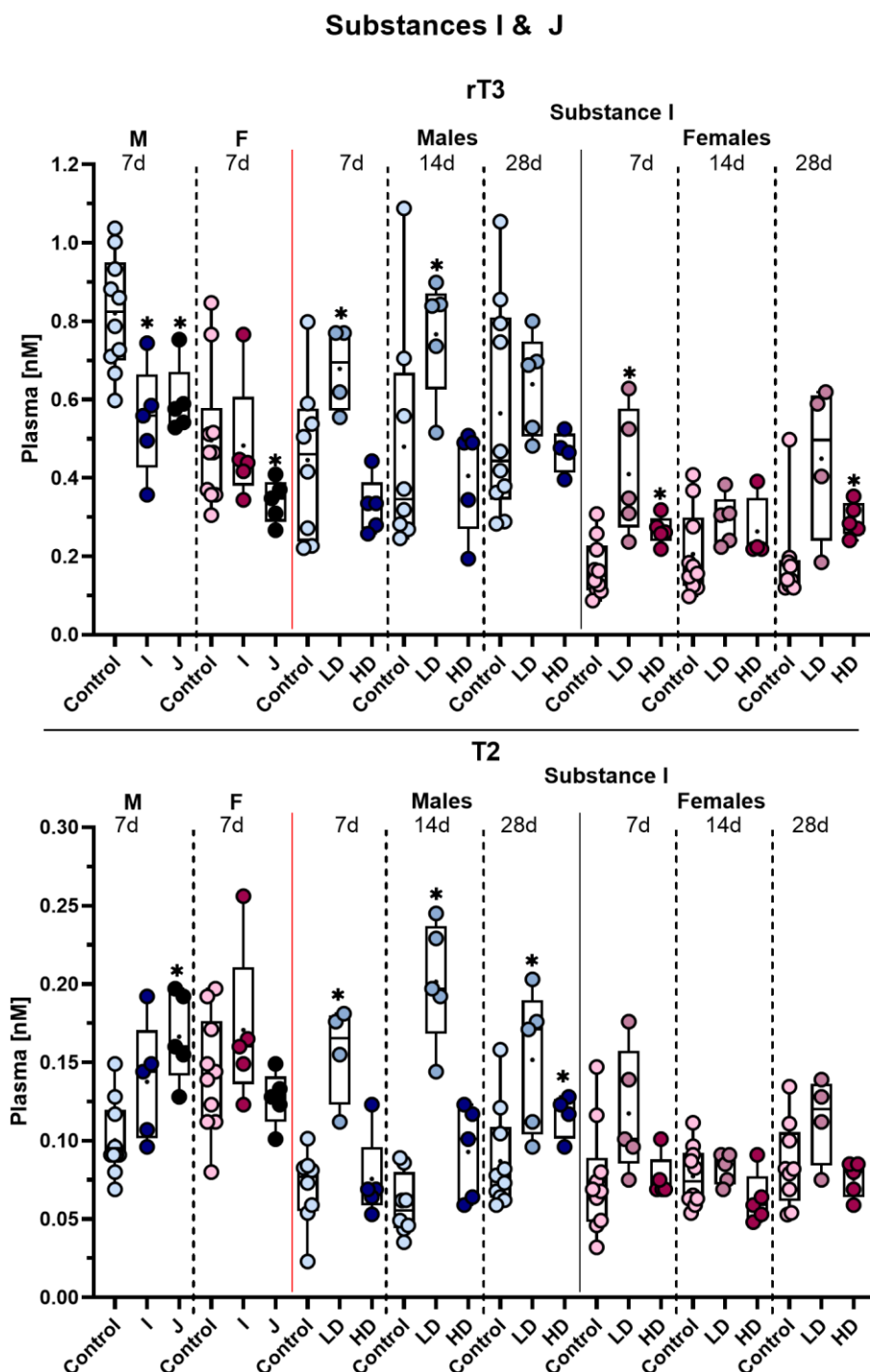


Figure 53. Results from rT3 and T2 analysis in rat plasma after exposure to substances I and J.

Rat plasma samples were received from two studies. The first one exposed the rats to either **substance I** or **J** for 7 days and the second one to **substance I** for a total of 28 days, but additionally after day 7 and 14 plasma samples were taken. A separation between the studies is shown by the first vertical red line. Results obtained from either males or females are shown above the box plots and further highlighted by the color of the dots (blue for males and red for females). Concentration is plotted on the y-axis as [nM] and on the x-axis control and substances of the first study as well as control or HD of the second study are listed. In the first study the black dots refer to **substance J** administration and the colored ones to **I**. The graph of **rT3** is ordered on top and below of **T2**. The asterisks refer to statistical significance: $* \leq 0.05$.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

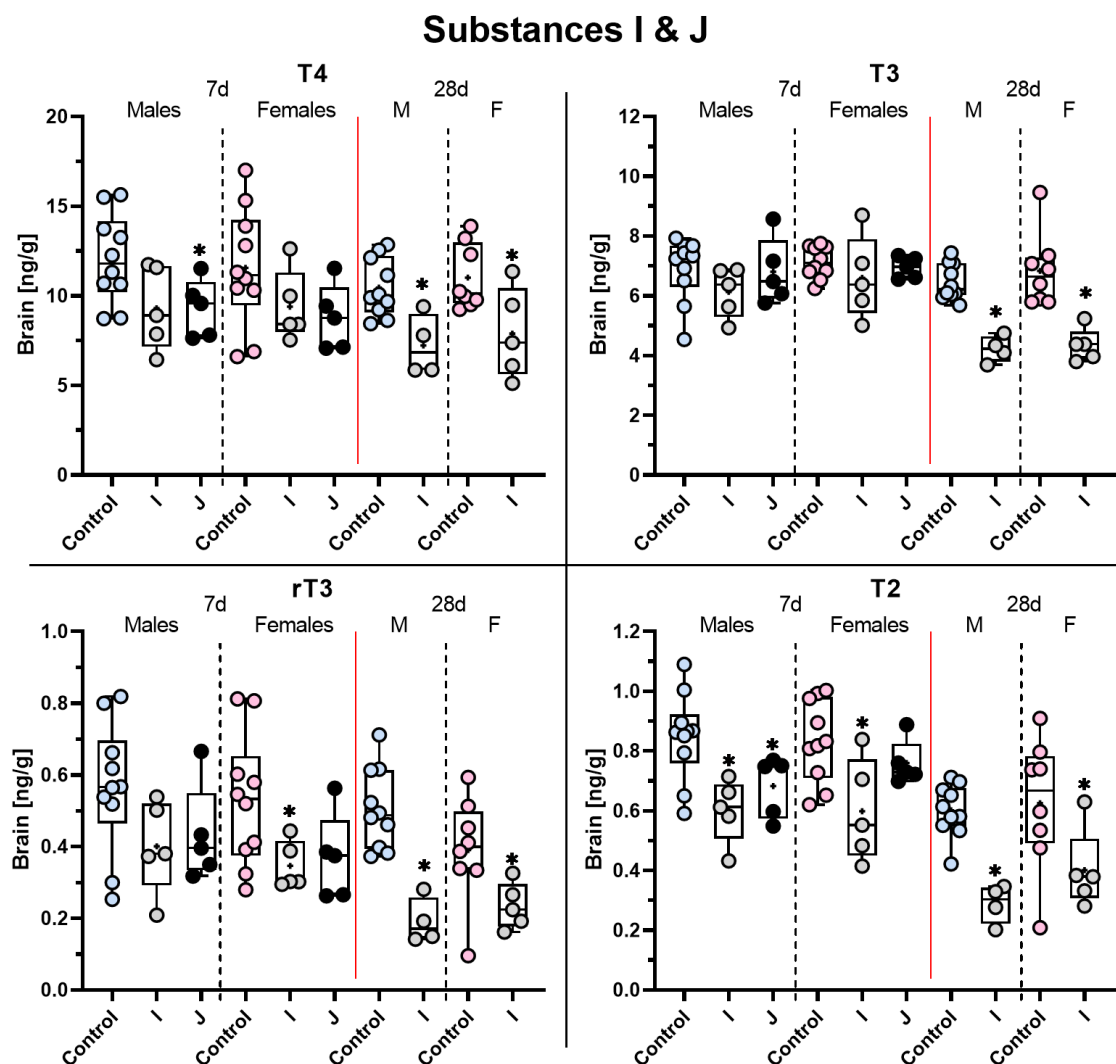


Figure 54. Effect of substances I and J on TH concentrations in adult rat brain.

The box plot graphs are plotted in an order from T4, T3, rT3 to T2. The vertical red solid line in each graph divides the data sets obtained from two different studies and males and females are separated by a dotted black line. Control and substance groups are mentioned on the x-axis whereas the color of the data points from the control groups indicate the results obtained from either males (blue) or females (red) which is also named above the box plots. Substances are differentiated by the dots in color light grey for **substance I** and black for **substance J**. Statistical significance is shown by the asterisks: $* \leq 0.05$.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.3.3 TH Alterations Caused by Organophosphates

Organophosphates (OP) are inhibiting the acetylcholinesterase (AChE) in the synaptic gap causing high concentration of acetylcholin followed by neurotoxicity⁷⁵. OPs possible TH-related MOAs are not clarified *in-vivo*⁷⁵. Some of the representative substances were investigated regarding their influence on TH concentrations in adult rat plasma and whole brain. Substances D and F were administered by gavage at doses of 150 mg/kg bw/d or 0.15 mg/kg bw/d. Administration of substance E was implied through diet at 65 ppm which corresponds to a dose of 5.2 mg/kg bw/d (250 g body weight and 20 g/d food consumption). TH analysis in rat plasma resulted for the most part in comparable concentrations for control and exposed groups (Figure 55). Only male rats exposed to substance D yielded significantly increased rT3 concentration and higher T2 concentration after substance E exposure. Substance F exposure on contrary led to significantly increased concentrations for T4, T3, and rT3 (Figure 56) in adult rat brain. In female rats exposed to substance E brain T4 and T3 were elevated where in males only T3 was increased. Notable, is the overall increased T2 concentration in rat brain from the study including substance F compared to the other study for which no plausible reason was found. Substance D administration did not cause significant TH alterations in adult rat brain.

The mentioned non-clarification of the suspected TH-related MOA by OPs is mirrored by the observed effects since they revealed a different pattern compared to the other insecticide classes. TH concentrations in rat plasma and brain were barely significant altered and if so, they were increased in both matrices.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

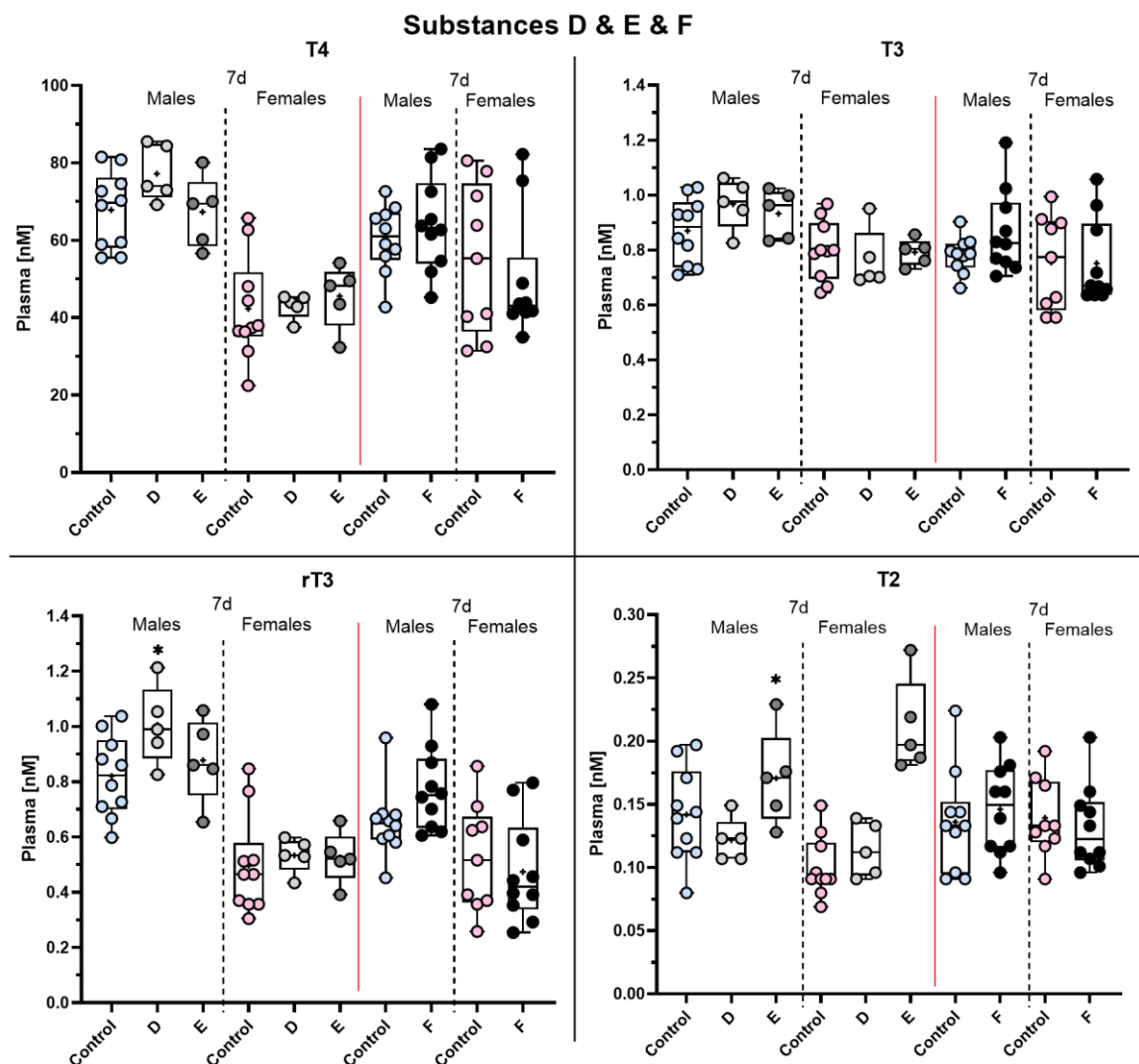


Figure 55. TH concentrations in rat plasma after substance D, E, or F administration.

Calculated concentration is scaled on the y-axis in [nM] and on the x-axis the control and substance groups. The data for the given sex is shown above the box plots and further indicated by blue (males) or red (females) colored dots. The compounds are distinguishable by light grey dots (**substance D**), dark grey dots (**substance E**), and black dots (**substance F**). **Substance F** was administered in a separate study than the other two substances which is emphasized by the red vertical line. The dotted black line separates data sets from males and females and graphs are ranked from left to right in subsequent order beginning with **T4**. Statistical significance is indicated by the asterisks: * ≤ 0.05 .

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

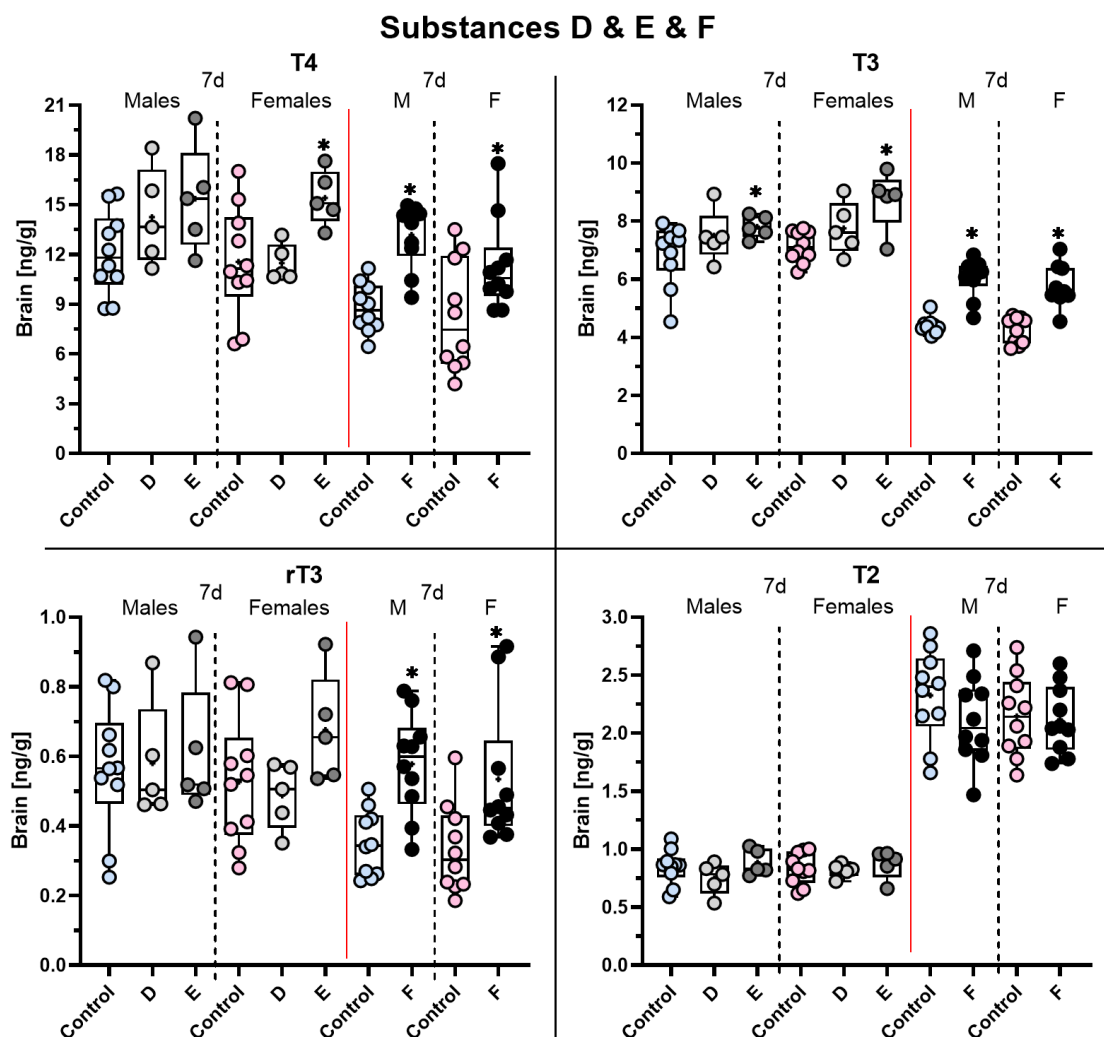


Figure 56. TH concentrations in adult rat brain after substance D, E, or F administration.

Calculated concentration is scaled on the y-axis in [ng/g] and on the x-axis the control and substance groups. The data for the given sex is shown above the box plots and further indicated by blue (males) or red (females) colored dots. The compounds are distinguishable by light grey dots (**substance D**), dark grey dots (**substance E**), and black dots (**substance F**). **Substance F** was administered in a separate study than the other two substances which is emphasized by the red vertical line. The dotted black line separates data sets from males and females and graphs are ranked from left to right in subsequent order beginning with **T4**. Statistical significance is indicated by the asterisks: * ≤ 0.05 .

5.7.4 Contrasting TH Plasma to Brain Data

For a better illustration of a potential time-dependent effect the respective brain data for each analyte were plotted against the corresponding plasma data for each individual animal for substances C, G, and I since data from two sampling days were available. As a reminder substance C is classified as pyrethroid, substance G as OC, and substance I as an organotin compound. In each graph control animal TH data for both sampling days were colored in the same bright blue and red colors but were distinguishable by their symbol; dots represented day 7 and squares sampling day 28. Data from exposed animals shared a star as symbol but differentiated by darkening of the color for which after 7 days the color was brighter than data

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

analyzed after 28 days. For each sampling day the symbols colored in blue refer to males and in red to females. Tendencies of separation from the control were highlighted by red solid lines or circles such as T4 concentration in plasma (y-axis) depicted an overall lower concentration in rats that were exposed to the HD of substance C than in controls, but no difference on x-axis (brain) was estimated as seen in Figure 57. The lowered rT3 concentration after 7-day exposure in males and the higher concentrations after 28 days in females (both in plasma) were highlighted by two red lines whereas brain values were similar on both sampling days. Like rT3, T3 concentration decreased in plasma after 7 days which was indicated by a red line. Therefore, it needed to be mentioned that T3 brain concentration in controls differed between the sampling days and that for analyte T2, data were available only after 28 days. T2 concentration in adult rat brain tended to be decreased after 28 days, however no statistical significance was detected. An exposure time dependency of substance G in plasma was detected for analytes T4, T3 and T2 which was more pronounced after 28 days, however for males from HD group after 28 days only two values were available as depicted in Figure 58. A shift to lower concentration in adult brain (x-axis) was more profound for T4 and rT3 after 28 days than after 7 days meaning that their concentration in rat brain decreased further with an extended exposure time. A time-dependent effect was further detected for all analytes from rats exposed to substance I regarding their concentration in adult rat brain which shifted to lower values with increasing exposure period visible in Figure 59 (left shift on x-axis). With regards to plasma concentration (y-axis) there was no time dependency detected since the analyte concentrations were mostly already decreased after 7 days or not significantly altered overall except for analyte T2 which concentration was increased.

In conclusion, no exposure dependent effect of substance C for neither of the analytes was identified since the alterations on both days were majorly similar. Substance G in both matrices as well as substance I in brain revealed a time dependent effect being more pronounced after 28 days.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

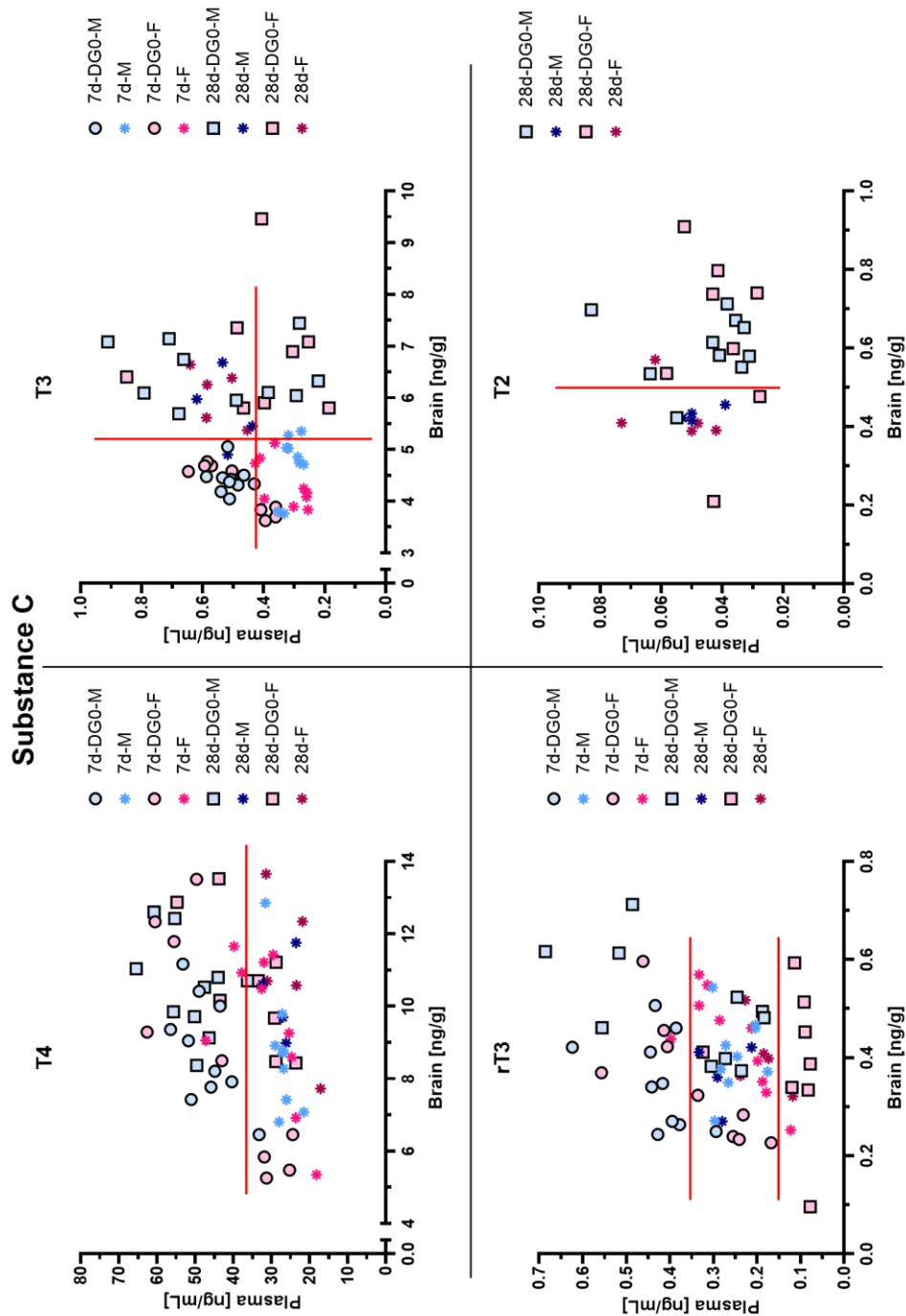


Figure 57. Contrasting of adult rat plasma and brain TH data for each individual animal after day 7 and day 28 administration of substance C. The y-axis represents plasma concentration in [ng/mL] and the x-axis is the corresponding brain concentration in [ng/g]. Data points after 7-day exposure are marked as dots for control animals and as lighter colored stars for exposed animals. The squares and darker colored stars are referring to the data set at day 28 respectively. Additionally, blue and red colors are used to differentiate between males and females as well as the red solid lines are inserted to indicate tendencies of alterations between control and dose groups.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

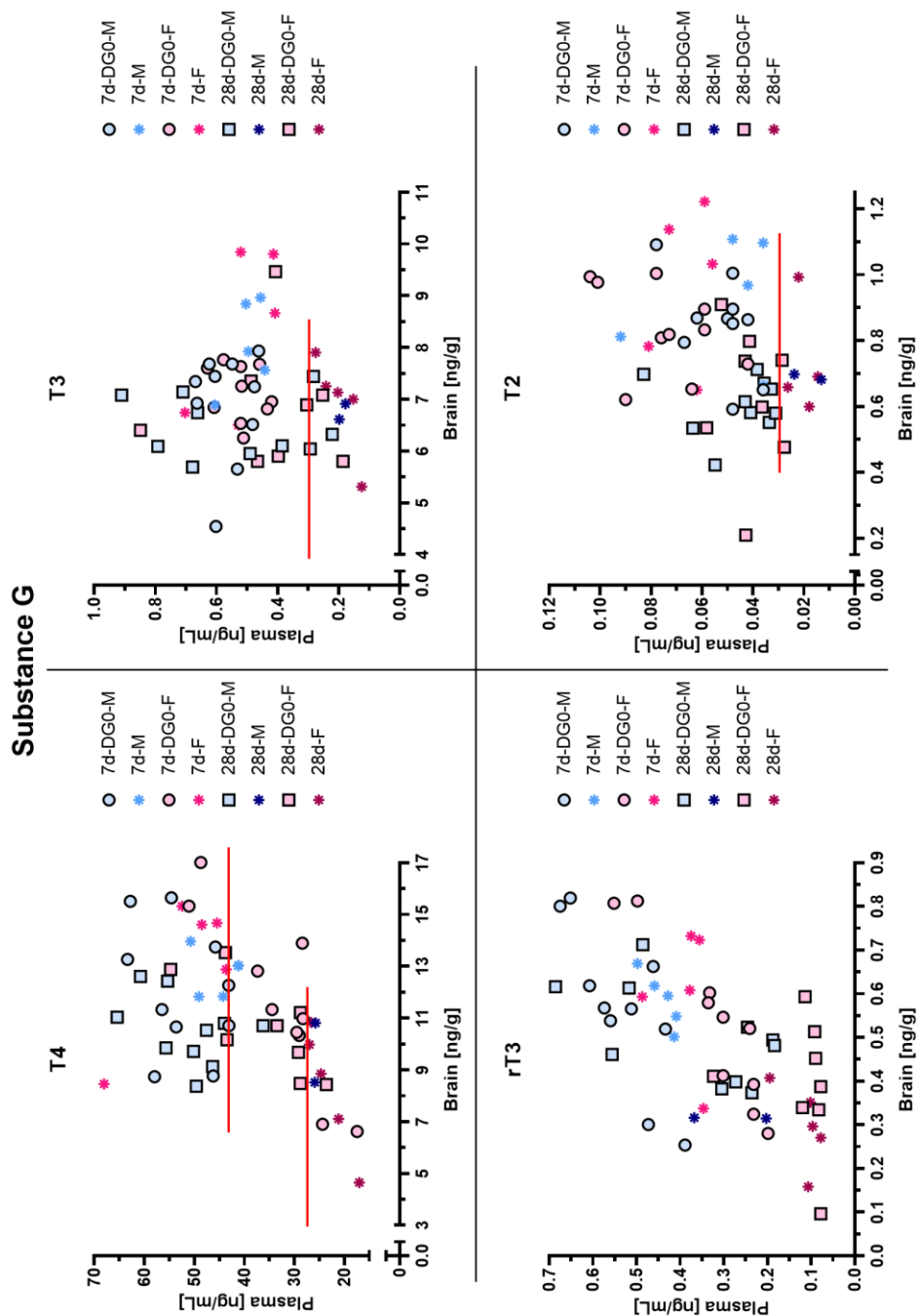


Figure 58. Contrasting of adult rat plasma and brain TH data for each individual animal after day 7 and day 28 administration of substance G. The y-axis represents plasma concentration in [ng/mL] and the x-axis the corresponding brain concentration in [ng/g]. Data points after 7-day exposure are marked as dots for control animals and as lighter colored stars for exposed animals. The squares and darker colored stars are referring to the data set at day 28 respectively. Additionally, blue and red colors are used to differentiate between males and females as well as the red solid lines are inserted to indicate tendencies of alterations between control and dose groups.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

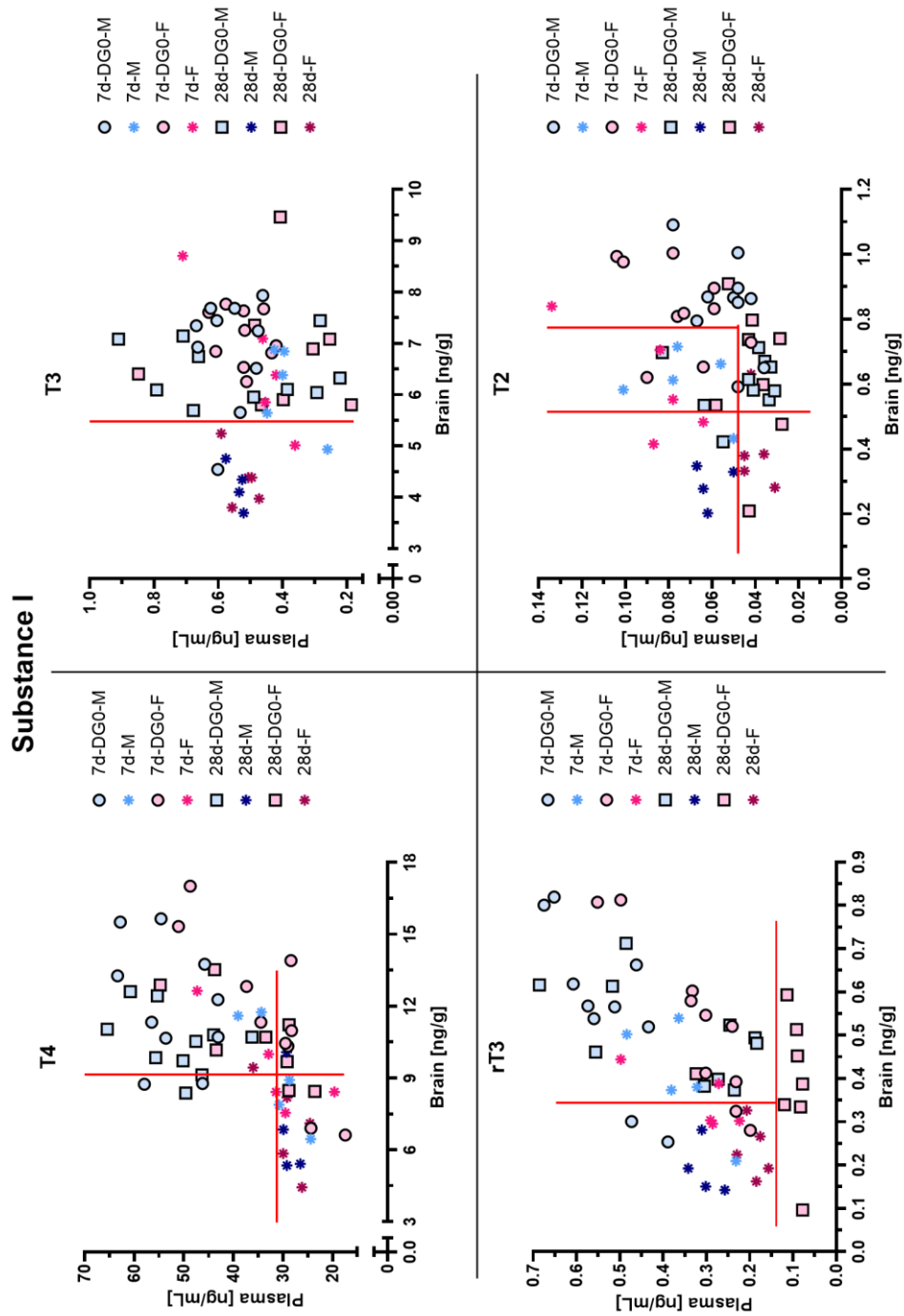


Figure 59. Contrasting of adult rat plasma and brain TH data for each individual animal after day 7 and day 28 administration of substance I. The y-axis represents plasma concentration in [ng/mL] and the x-axis the corresponding brain concentration in [ng/g]. Data points after 7-day exposure are marked as dots for control animals and as lighter colored stars for exposed animals. The squares and darker colored stars are referring to the data set at day 28 respectively. Additionally, blue and red colors are used to differentiate between males and females as well as the red solid lines are inserted to indicate tendencies of alterations between control and dose groups.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

5.7.5 Final Comparison of TH Alterations Caused by Different TH-Related MOAs

A summary of the calculated alterations between control and HD group is enumerated for each analyte per substance and per sampling day and per sex in Table 23. The value was calculated for statistically significant alterations whereas the others were marked non-significant (ns). A slash was added in the given cell when no samples were available such as seen on day 28 for MMI for which no control animals at day 28 were available. No HD data was further available for Phenyl Isothiocyanate at day 14. As well as only four of the blinded substances were applied for 28 days whereas the remaining for 7 days.

Substances PTU, MMI, and Aroclor 1254 exhibited the strongest decline between 84-99 % on days 14 and 28 for all three analytes. HD group of Aroclor1254 however obtained a decline of around 90 % for analytes T4 and rT3 whereas a decrease of T3 concentration stayed within 42-52 % at the sampling days. Decline of T3 was similar to its decline after Vinclozolin exposure which yielded a 42 % decline after 28 days in males and 58.1 % after 14 days in females for which day 28 was not statistically significant. On day 7 males and females yielded a similar reduction of T3 of around 25 %. Concentration of T4 and rT3 was more profoundly altered in females showing a decrease of around 70 % on sampling day 14, however alterations on day 7 and 28 were not significant. In males these two analytes were significantly reduced on day 7 and day 28 by around 56 % for T4 and 39 % to 47 % for rT3. To sum up, PTU and MMI as TPO inhibitors were the most potent substances to decrease TH concentration in plasma, however the liver enzyme inducer Aroclor 1254 showed a very similar pattern at least for the decrement in T4 and rT3 concentrations. On contrary, T3 concentrations were not detected to decrease as sharply as after exposure to PTU or MMI (around -50 % vs. - 90 %). T3 decrease due to Aroclor 1254 exposure was in a similar range as after Vinclozolin administration which were both around 50 %. Vinclozolin as well caused a decrease in T4 and rT3 concentrations of around 56 to 74 % which was not significant anymore for females after 28 days. Of all substances examined, ETU affected TH concentration the most different compared to the other substances. Magnitude of decreased T4 and rT3 concentrations were in a similar range as for Vinclozolin, however T3 concentration increased after ETU exposure. This effect set ETU apart from PTU and MMI even though all three are interfering directly with TH homeostasis.

A stronger effect in particular in male rats than for Vinclozolin was detected for plasma TH-binding protein substances TBBPA and PFHxS. T4 concentrations after exposure to either substance were reduced by up to 64 % or 86 % respectively which was mirrored in detected rT3 concentrations. Interestingly, only these two substances showed a weakening of reduction

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

over time. T2 concentrations in plasma of control animals were already close to LOQ wherefore substances causing a decline in total TH concentration as shown here could cause T2 concentration to fall below the LOQ level which explained the majorly few data points in the presented figures. T1 concentrations were only detected in females but not consistently throughout the project.

Between all insecticides, substances B, C, G, I, and J caused a decline in plasma T4 concentration in females and males which was further caused by H and K in males but in females T4 increased after exposure to substance H. In females, substance K led to a non-significant effect of T4 as well as substances D to F in males and females. Substance B increased the concentrations of the remaining analytes in both sexes whereas substances G, J, and K decreased rT3 and T3 concentrations significantly. The OP substances D, E and F mostly did not significantly affect TH concentrations in plasma. On contrary in rat brain only T2 was unaltered whereas concentrations of T4, T3, and rT3 increased majorly after exposure to substance F and some analytes also after administration of substance E. Majority of TH concentrations were not significantly altered in brain when exposed to the mentioned doses of substances B, C, D, J, G (7-day exposure), H, and K. For individual analytes the respective concentration in brain was affected such as e.g. substance K significantly decreased T4 and T2 concentrations in males but significantly increased T3 concentration in females. Another example was the time-dependent effect of substances G (OC) and I (Organotin) which significantly lowered T4, T3, and rT3 concentrations after 28 days but not after 7 days. Each blinded substance is categorized in a different insecticide class in which they were sorted in Table 23. Regarding their potential to reduce TH concentrations in plasma, substances C, G, and I yielded similar percentual reductions for T4 in males of around 44 to 50 % after day 28 which were similar to the percentual reduction caused by ETU and Phenyl Isothiocyanate. Only substance G caused significant T3 reductions with tendency to strengthen over time which was slightly stronger at day 28 compared to Aroclor 1254 or Vinclozolin. Substances B, C and I significantly increased rT3 concentrations in females and substance B also in males. Most blinded substances administered for 7 days did not cause significant alterations for none of the TH analytes except for substances J, and K.

All in all, as expected the strongest TH reductions in plasma were detected for substances directly interacting with the TH system. An exemption is liver enzyme inducer Aroclor 1254 causing similar reduced T4 and rT3 concentrations as PTU and MMI. For the most part the substances from the different insecticide classes outlined a similar TH alteration pattern in adult rats. This pattern consisted of TH concentrations being predominantly altered in rat plasma.

5.7 Alteration of Total TH Concentrations in Rat Plasma Caused by Different MOAs

Significantly changed TH concentrations in whole brain analysis were detected for most substances after 28 days. Only substance A did not modify any of the analytes in neither matrix at the administered dose which was unlike the other two blinded substances from that insecticide class.

Table 23. Significant TH concentration alterations at three sampling days in HD animals compared to control animals.

The change in concentration for analytes **T4**, **T3**, and **rT3** in the HD group is listed at three sampling days (7, 14, and 28) for each substance as well as for male and female rats. The first three substances PTU, MMI, ETU and Phenyl Isothiocyanate are involved in a direct thyroid MOA whereas Aroclor 1254, Vinclozolin, TBBPA and PFHxS influence THs indirectly. For 4 blinded substances of the tested insecticides, all sampling days were available which were substances B, C, G, and I. All other blinded substances were administered for 7 days. Only the percentage was mentioned when the change was statistically significant, otherwise it is marked with “non-significant” (ns). At certain sampling days no values were obtained and therefore the given cell is marked with a slash.

((mean(DG0)-mean(HD))/mean(DG0))*100 [% Increase/Decrease TH Conc. from Control]			Male Rats			Female Rats		
	Substance	Analyte/ Sampling Day	7 [%]	14 [%]	28 [%]	7 [%]	14 [%]	28 [%]
TPO Inhibitors	PTU	T4	-65.5	-97.9	-98.7	ns	-97.7	-97.0
		T3	-68.9	-90.7	-89.8	ns	-84.9	-78.3
		rT3	ns	-90.1	-91.7	+184.6	-87.3	-84.6
	MMI	T4	-86.1	-98.1	/	-68.0	-96.7	/
		T3	-81.6	-96.0	/	-54.0	-93.7	/
		rT3	-87.4	-99.2	/	-77.6	-90.0	/
	ETU	T4	-53.5	-56.6	-49.2	ns	-47.4	-38.6
		T3	+57.3	ns	+32.6	+48.4	+77.4	+139.3
		rT3	-70.6	-61.8	-45.8	ns	ns	ns
NIS Inhibitor	Phenyl Isothiocyanate	T4	-61.6	/	-43.0	-32.5	/	ns
		T3	-49.4	/	-41.7	ns	/	ns
		rT3	-71.7	/	-70.1	-62.5	/	ns
Liver Enzyme Inductors	Aroclor 1254	T4	-85.8	-91.4	-93.1	-75.6	-87.4	-87.8
		T3	-35.7	-48.7	-42.1	ns	-42.1	-51.8
		rT3	-84.4	-91.4	-95.7	-63.1	-85.2	-86.5
	Vinclozolin	T4	-56.4	-50.6	-56.1	ns	-74.3	ns
		T3	-25.7	-24.4	-42.0	-24.8	-58.1	ns
		rT3	-39.7	-35.7	-47.1	ns	-69.2	ns
Binding to Plasma TH-Binding Proteins	TBBPA	T4	-74.3	-72.0	-63.7	-46.8	-43.6	-74.3
		T3	-53.1	-46.4	-38.2	ns	-40.3	ns
		rT3	-74.2	-71.0	-62.7	ns	ns	ns
	PFHxS	T4	-89.6	-89.8	-86.0	-52.3	-57.8	-49.7
		T3	-64.6	-62.0	-59.6	ns	-28.2	ns
		rT3	-85.6	-86.3	-83.2	-73.0	-69.2	-57.8
Pyrethroids	A	T4	ns	/	/	ns	/	/
		T3	ns	/	/	ns	/	/
		rT3	ns	/	/	ns	/	/
	B	T4	ns	-21.2	-25.2	ns	-52.7	ns
		T3	+70.6	+37.6	+61.9	+40.5	ns	ns
		rT3	+50.8	+71.3	+112.4	+111.5	ns	ns
	C	T4	-46.3	-48.0	-46.6	-26.7	-43.7	ns
		T3	ns	ns	ns	ns	ns	+45.2
		rT3	ns	ns	ns	+71.1	ns	+102.4
OP	D	T4	ns	/	/	ns	/	/
		T3	ns	/	/	ns	/	/
		rT3	+22.5	/	/	ns	/	/

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

((mean(DG0)-mean(HD))/mean(DG0))*100 [% Increase/Decrease TH Conc. from Control]			Male Rats			Female Rats		
	E	T4	ns	/	/	ns	/	/
		T3	ns	/	/	ns	/	/
		rT3	ns	/	/	ns	/	/
	F	T4	ns	/	/	ns	/	/
		T3	ns	/	/	ns	/	/
		rT3	ns	/	/	ns	/	/
OC	G	T4	ns	-30.6	-49.5 (n=2)	-17.8	ns	-34.4
		T3	-41.3	-35.9	-65.3 (n=2)	-42.8	-52.9	-55.5
		rT3	ns	ns	ns	ns	ns	ns
	H	T4	-14.0	/	/	ns	/	/
		T3	ns	/	/	ns	/	/
		rT3	ns	/	/	ns	/	/
Organotin	I	T4	-46.2	-52.5	-43.7	-35.5	-48.0	ns
		T3	ns	ns	ns	ns	ns	ns
		rT3	ns	ns	ns	+57.5	ns	+55.2
	J	T4	-30.6	/	/	-27.8	/	/
		T3	-19.6	/	/	-22.9	/	/
		rT3	-27.1	/	/	-31.3	/	/
Phenyl Pyrazole	K	T4	-41.9	/	/	ns	/	/
		T3	-25.7	/	/	-20.0	/	/
		rT3	-33.0	/	/	ns	/	/

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

An animal study with Wistar rats was conducted to determine the effect of the common rat diet (Iodide-supplemented) compared to a non-Iodide-supplemented diet. The received samples are listed in Table 11. Iodide concentration in the control group yielded 1 mg/kg Iodide and in the non-supplemented (NS) diet around 0.16 mg/kg. After 4 weeks of receiving either diet the rats were mated and the TH concentrations in particular in the offspring brain and plasma were investigated. TH concentrations in plasma were analyzed in the parent animals within the four weeks after day 7, 14, and day 28. The respective results are depicted in Figure 60 and overall, no statistical significant difference in TH concentrations between the diets were established except for a slight decrease of rT3 in males after 7 days which was undetectable after 14 days and 28 days. Lastly, only in adult female rats T1 concentration were identified.

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

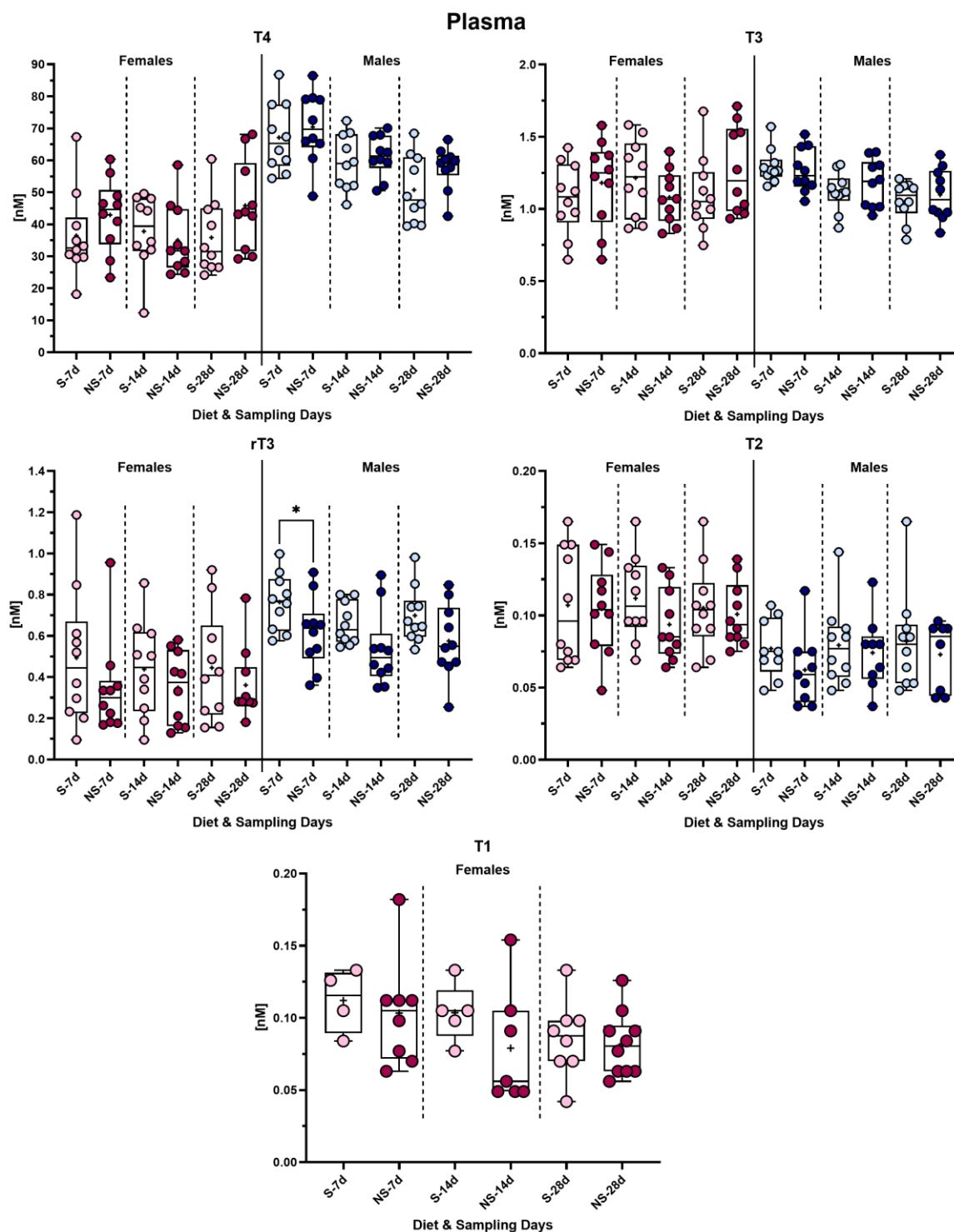


Figure 60. Plasma TH concentrations of adult rats after feeding of Iodide-supplemented or non-supplemented diet.

On the top left results of TH analyte **T4** are presented, and the graphs are further sorted by number of structurally containing Iodide. The solid line in every graph divides female and male data sets whereas visually they can be further identified by either red or blue colored dots. The darker colored dots imply data sets from rats receiving the non-supplemented ('NS') diet. Additionally on the x-axis besides the diet, the sampling days are mentioned for each box plot which were at days 7, 14 and 28. Statistical significance is estimated by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

Plasma and whole brain sampling in the offspring were obtained on PND4, 21 and 30. On contrary to the remaining days, plasma was pooled from culled pups on PND4 per litter and per sex wherefore not a consistent number of animals per litter could be received. However, on PND21 and 30 mostly 10 individual animals per sex and group were received (n=10) except for females in the control group on PND21 (n=7) and males in the non-supplemented group on PND30 (n=9). The whole brain samples on all investigated days were obtained from the same individual animals as the plasma samples on PND21 and 30. TH analysis in pup plasma is summarized in the graphs per TH analyte in Figure 61. The data sets were sorted per age starting with PND4 as well as female and male results were shown pairwise which are additionally recognizable by red and blue dots. Further, the darker colored dots ensemble results from animals in the non-supplemented ('NS') group. Statistically significant increase of T4 concentration in all animals were assessed on PND21 and PND30. On PND30 further significant increase was detected for rT3 as well as for females on PND30 for T2. TH analyte T3 yielded steady concentrations on all days as well as between the diet groups. The corresponding TH concentrations determined from whole brain analysis of the same individual animals are presented in Figure 62. On average 10 animal brains were received per group and sex, however some fluctuation occurred such as supplemented females on PND4 were eleven (n=11), but males only one (n=1) and on PND21 seven females were available (n=7). In the NS group of males on PND4 twelve animals were included (n=12), whereas one male less than the average was received on PND30 (n=9). In Figure 62 the previously established observation of increasing T4, T3, and T2 concentrations but decreasing rT3 concentration from PND4 to PND21 were detectable. Additionally, this data highlighted the assumption that on PND21 a peak concentration of analytes T4, T3, and T2 was manifested in the developing rat brain due to the detection of lowered concentrations on PND30 which were similar to the established concentrations in adult rats. On contrary, rT3 peak concentration was on PND4. Regarding the influence of the received diets in the developing rats, a significant increase in T4, T3, and rT3 concentrations were measured on PND30 as already detected in the respective plasma samples for analytes T4 and rT3.

All in all, non-supplementation of Iodide in the diet did not lead to decreased TH concentrations such as it would be expected for Iodide deficient diet. On PND30 significant increased concentrations of mostly T4 and rT3 were established in plasma and brain, however in adult rats (parental animals) no changes were detected.

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

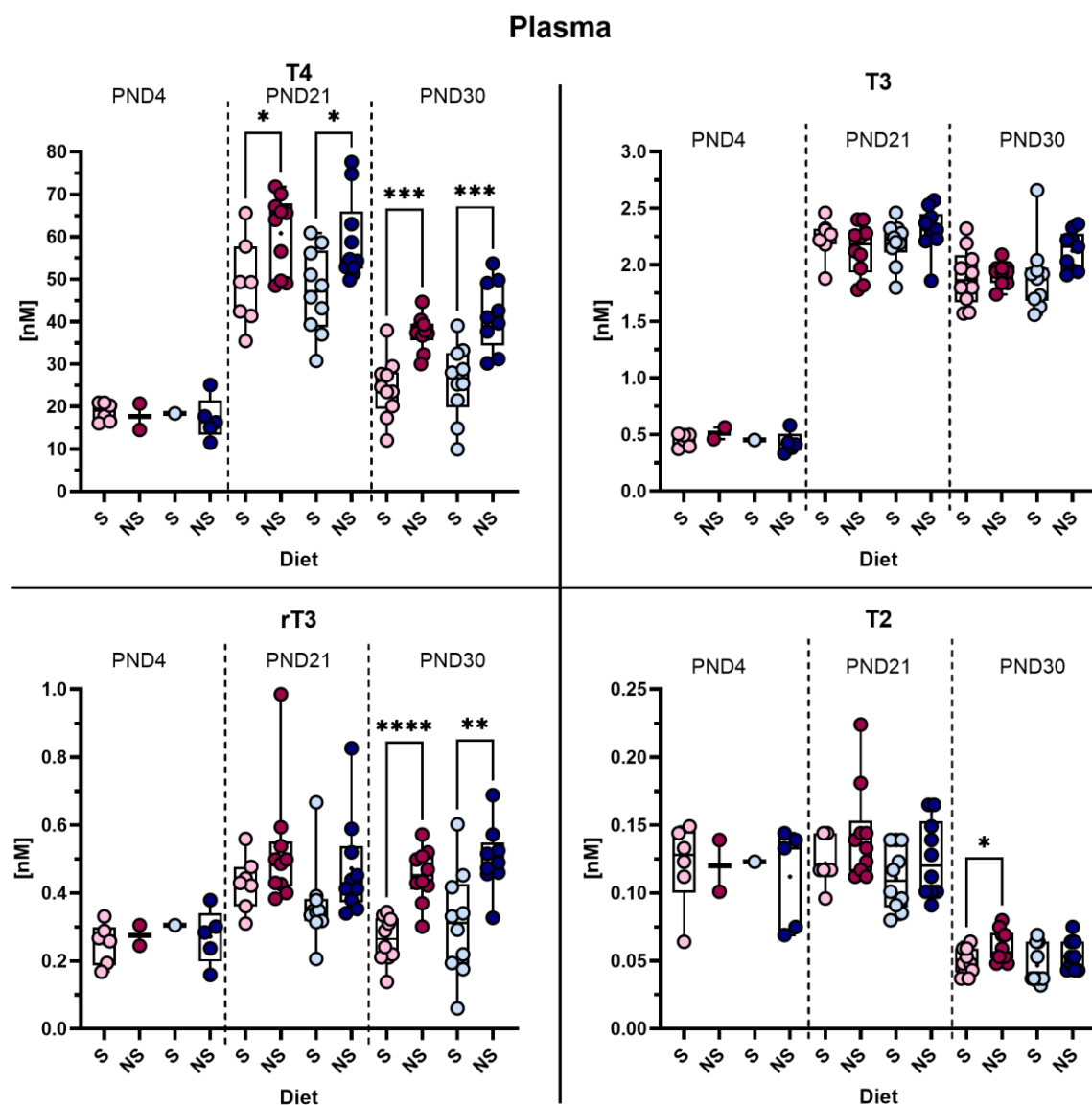


Figure 61. TH concentrations in pup plasma on PND4, 21 and 30 after receiving different Iodide-rich diets. The graphs are ranked in order from T4 to T2 starting from the top left. The red and blue dots represent data sets from female or male pups respectively whereas the darker color indicates the data set from the non-supplemented group. On the x-axis the letter 'S' defines the control group receiving supplemented diet and the non-supplemented diet is abbreviated to 'NS'. The results from the different days are separated by dotted lines and the given sampling day is written above the box plots. The TH plasma concentrations are depicted in [nM] on the y-axis. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.8 Influence of Iodide-Supplemented and Non-Supplemented Diet on TH Concentrations in Developing Rats

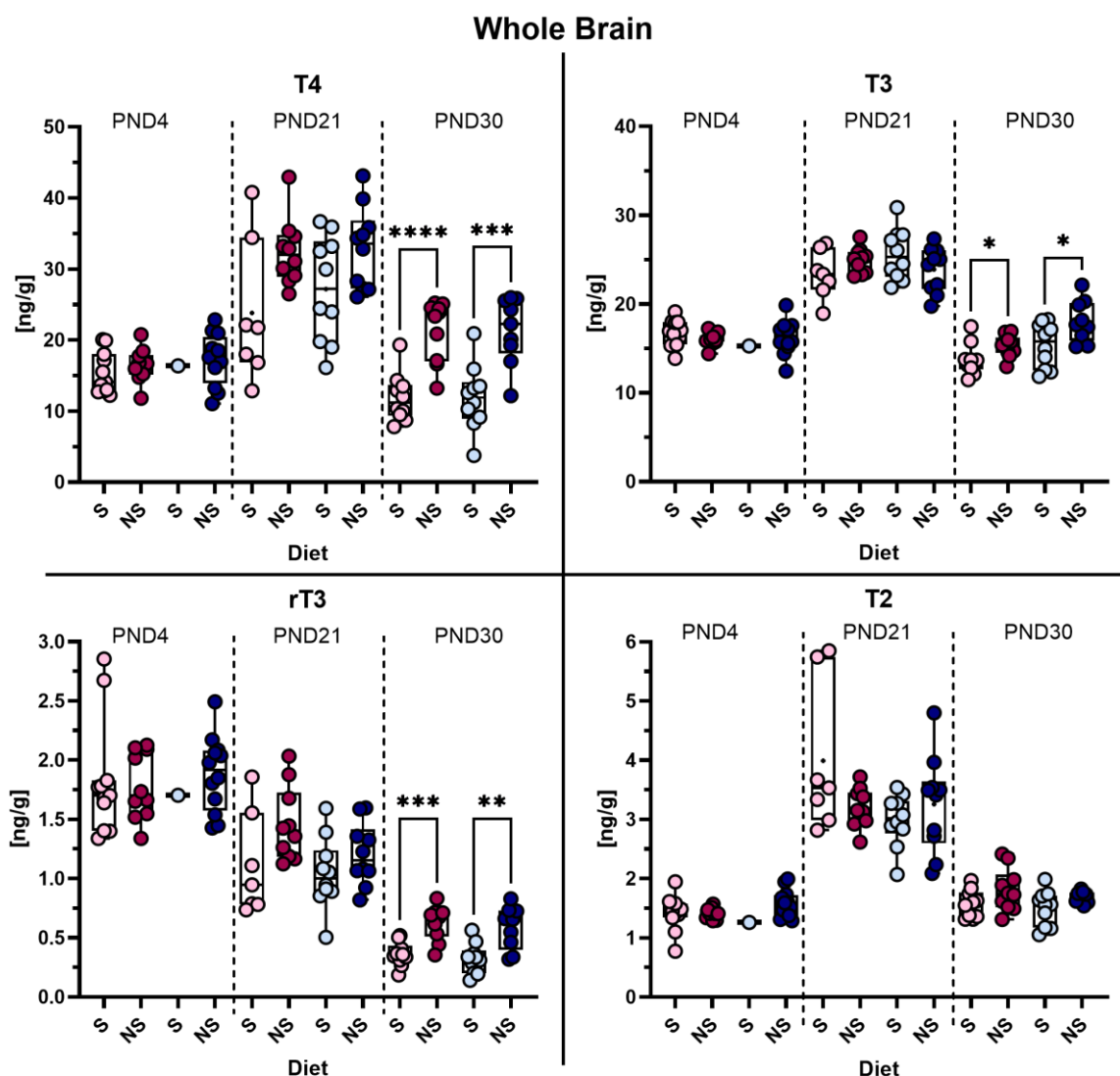


Figure 62. TH concentrations in pup brain on PND4, 21 and 30 after receiving different Iodide-rich diets. The graphs are ranked in order from T4 to T2 starting from the top left. The red and blue dots represent data sets from female or male pups respectively whereas the darker color indicates the data set from the non-supplemented group. On the x-axis the letter 'S' defines the control group receiving supplemented diet and the non-supplemented diet is abbreviated to 'NS'. The results from the different days are separated by dotted lines and the given sampling day is written above the box plots. The TH brain concentrations are depicted in [ng/g] on the y-axis. Statistical significance is presented by the asterisks: * ≤ 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

5.9 Alternative Test Methods to Quantify TH

5.9.1 TH Content in the Developing Zebrafish

5.9.1.1 Age-Dependent TH Concentration in Zebrafish Embryos to Larvae

The first experiment was conducted to estimate how many pooled zebrafish embryos/larvae were necessary to reach sufficient peak signal intensity of the analytes. Two different amounts (n=25 & 50) of pooled zebrafish larvae were analyzed regarding TH concentrations at timepoint 120 hpf and the samples were purified as detailed in section 4.1.7. A deviation from the description in section 4.1.7 was that for the first experiment two types of sample preparation were examined for their TH extraction. The five received samples were divided into three samples which were extracted as mentioned prior (referred to as 'A') and into two samples that additionally received filtration and washing steps prior to the final evaporation (referred to as 'B'). In Figure 63A the nominalized TH concentration per one larva and for each sample preparation procedure is illustrated. Both sample preparation procedures as well as both number of pooled zebrafish larvae yielded almost identical T4 and T3 concentrations. Notable was the higher standard deviation between the duplicates "50 x ZF 'B'" for T4. The corresponding mean analyte areas are depicted in Figure 63B which were consistently lower for T4 and T3 in samples that were prepared following procedure 'B'. Therefore, samples from further experiments were handled as described in section 4.1.7 and in this paragraph referred to as 'A'. To ensure analyte peak signal intensity in the forthcoming experiments, the number of 50 x pooled zebrafish embryo/larvae was selected to proceed with. In the second experiment different sampling days were investigated regarding the TH concentrations in the developing zebrafish embryo. The sampling days were equal to the age of the embryo and the value from the first experiment was included for the last timepoint (120 hpf). In Figure 64 total pooled concentrations of T3 (n=3) with increasing embryo age are presented. T3 concentration decreased over time and reached around one third of the value from 24 hpf at 120 hpf. In the second experiment T4 could not be sufficiently detected.

5.9 Alternative Test Methods to Quantify TH

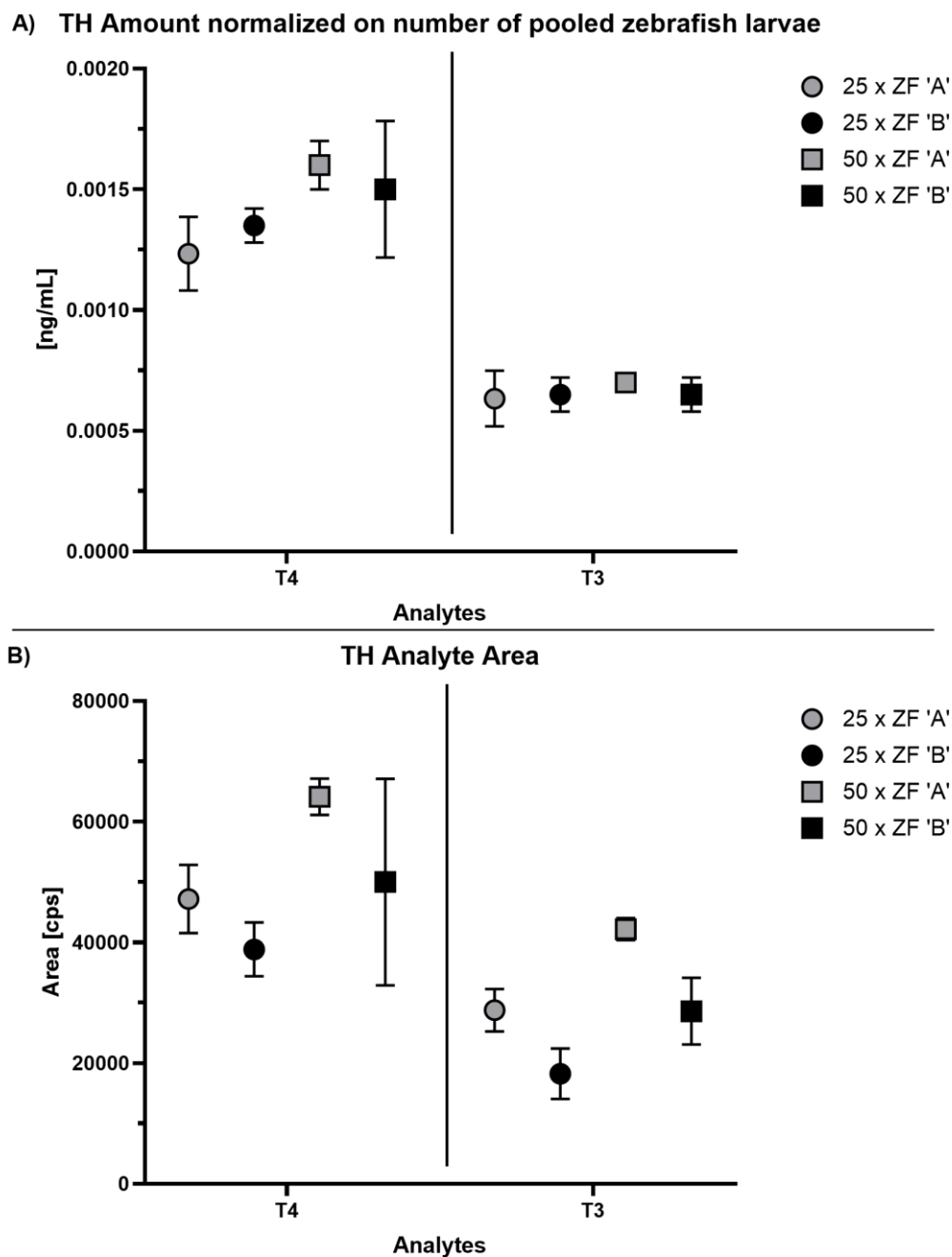


Figure 63. Results from first experiment analyzing 120 hpf-old zebrafish larvae.

For both graphics the illustrated symbols distinguish between the results from 25 pooled larvae (circle) and 50 pooled larvae (square), and the colors reflect sample preparation 'A' (grey), and sample preparation 'B' (black) as described above. A) On the left the nominal TH concentrations per larva is plotted on the y-axis and on the x-axis the analytes that could be detected (T4 and T3). B) Each mean peak area of T4 and T3 [cps] is plotted for the detected analytes.

5.9 Alternative Test Methods to Quantify TH

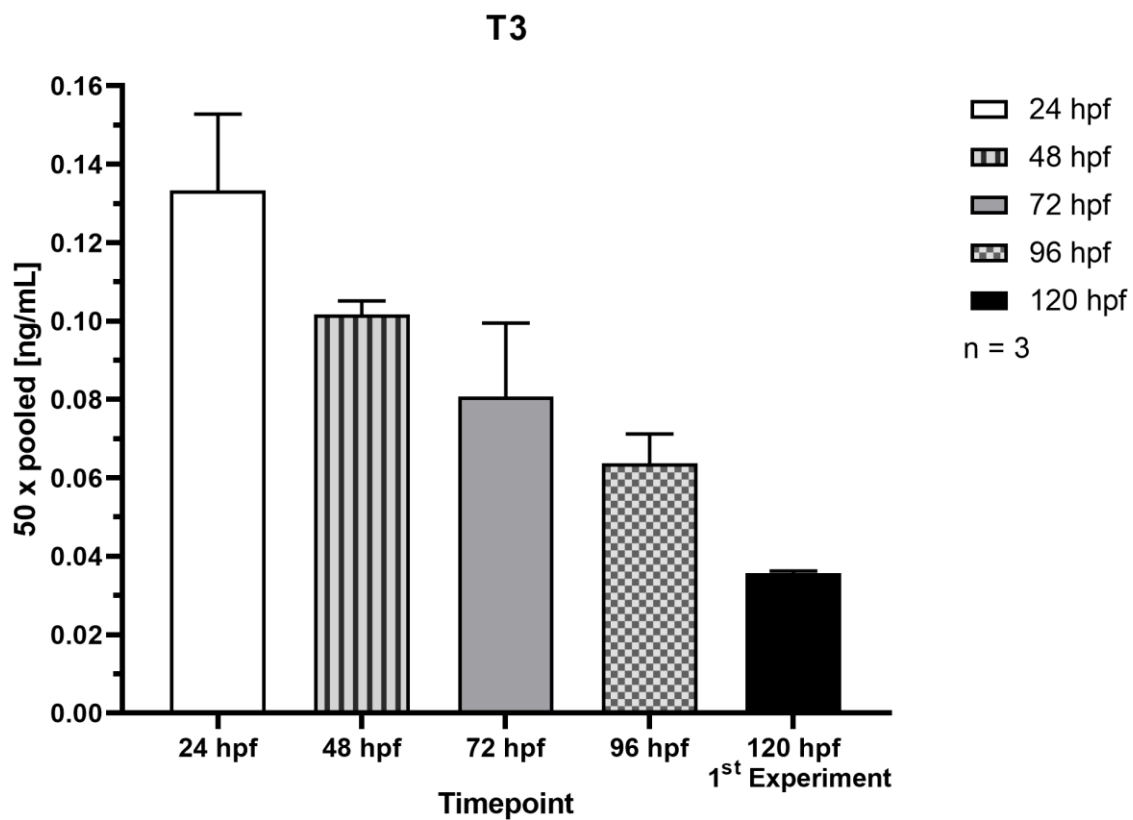


Figure 64. T3 concentrations in the developing zebrafish embryo at different sampling days.

Each timepoint consists of three biological replicates whereas each one comprises of 50 x pooled zebrafish embryos. Total concentrations in the pooled amount are plotted on the y-axis and on the x-axis the sampling days which is equal to the age of the embryos.

5.9 Alternative Test Methods to Quantify TH

5.9.1.2 Time-Dependent Alteration of TH Concentrations after PTU Exposure

In further experiments, zebrafish embryos were exposed to different PTU concentrations over different incubation periods to estimate its influence on TH concentrations. A total of six concentrations were tested: 0, 325, 487, 731, 1640, and 3700 μM which were exposed to the zebrafish embryos for periods of either 24, 48, 72, or 96 hpf. For each concentration at each incubation time four replicates were generated whereas one replicate was missing for concentration 731 μM after 72 hpf and 96 hpf respectively. From the above shown results it was concluded to analyze 50 pooled embryos to guarantee sufficient analyte peak intensity, however, this number of pooled embryos was not possible to obtain for every replicate in this experiment due to a higher loss of surviving embryos after fertilization. Therefore, the calculated concentration was divided by the number of pooled embryos received in the respective sample to finally normalize every sample resulting in a concentration per one embryo which guaranteed better comparability between the samples. The minimum number of embryos received were 25 pooled embryos per sample. Results from this experiment are visualized in Figure 65 in which the top graph includes results for T4 and the graph below for T3. The different incubation times are visually separated by black dotted lines which are listed on the x-axis. The various colored box plots refer to the respective PTU concentration which is highlighted in the legend. T4 concentrations in control embryos seem to increase with age in particular from 74 hpf to 96 hpf an increase from 0.110 ± 0.020 pg/embryo to 0.314 ± 0.145 pg/embryo was estimated. This refers to the onset of T4 synthesis which has been detected at around 70 to 80 hpf¹⁴¹. Thus, significant changes in T4 concentrations of zebrafish embryos exposed to PTU were detected after incubation for 96 hpf because T4 concentrations in the corresponding exposed groups were similar at all incubation periods but the control group at 96 hpf increased compared to the other control groups. On contrary, overall control T3 concentrations decreased with increasing age/incubation period from 0.398 ± 0.152 pg/embryo at 24 hpf to 0.111 ± 0.035 pg/embryo at 96 hpf. No significant T3 alterations were observed in none of the given PTU concentrations after neither incubation period.

5.9 Alternative Test Methods to Quantify TH

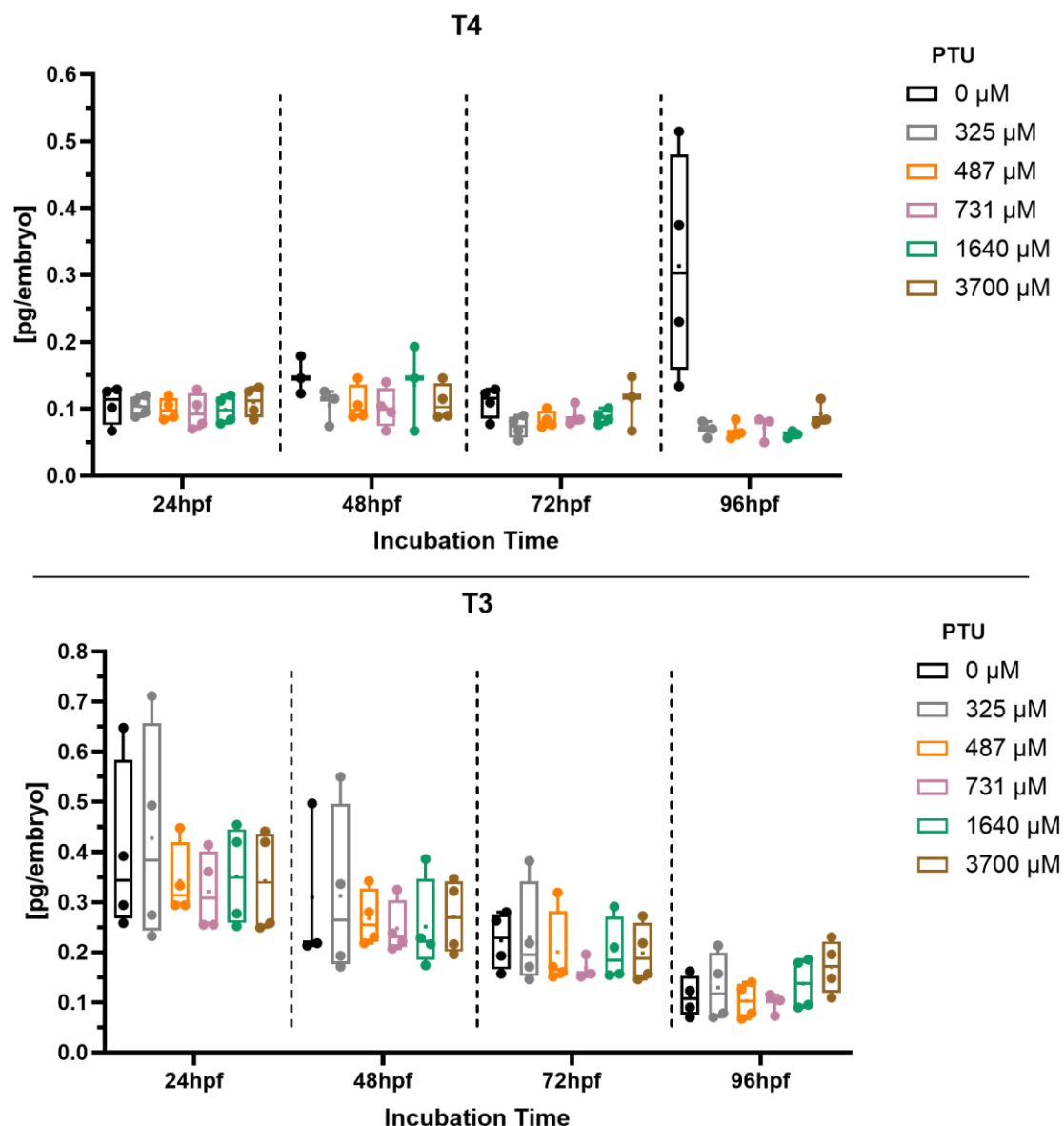


Figure 65.Effect of PTU exposure on TH concentrations in whole zebrafish embryos.

Calculated concentration was normalized onto one embryo which is plotted on the x-axis. On the y-axis the four incubation periods are listed (24, 48, 72, and 96 hpf) and divided by black dotted lines. In each incubation period, five different PTU concentrations were administered along with one control group receiving no treatment. Different PTU concentrations are highlighted by the colored box plots whereas control group is painted black, 325 μM grey, 487 μM orange, 731 μM pink, 1640 μM green, and 3700 μM brown. Besides that, the top graph portrays results for total T4 and the bottom graph for total T3.

5.9 Alternative Test Methods to Quantify TH

5.9.2 Detection of DIO1 Inhibition in Human Liver Microsomes Using Online-SPE-LC-MS/MS

5.9.2.1 Several Substances Inhibit DIO1 Indicated by a Change in rT3 and T2 Concentrations

DIO1 conversion of rT3 to T2 in human liver microsomes was analyzed after exposure to various substances which were initially selected to be tested within a joint research program focusing on establishing a reliable *in-vitro* test battery for the TH system initiated by The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM). Within this joint research program, the DIO1 Sandell-Kolthoff (DIO1-SK) assay has been further improved and pre-validated for human liver microsomes^{129, 130}. The DIO1-SK assay readout is based on the photometric measurement of the color change of cerium solution from yellow (Ce^{4+}) to colorless (Ce^{3+}) catalyzed by free Iodide in the solution¹⁴². This reaction is susceptible to interferences by other reducing substances as for example ferrous ion or thiocyanate¹⁴², but also to Iodide-containing substances for which it is not distinguishable whether the free Iodide results from the substance or the actual rT3 conversion. Therefore, a new test cohort of the same testing substances were established to confirm results obtained using the DIO1-SK assay and secondly to yield results for SK-interfering substances. Each substance was tested at two different experimental days reflecting two biological replicates and every concentration group as well as control groups consisted of three technical replicates. The first experimental part meaning incubation of the substances with the human liver microsomes were conducted at BASF department for Experimental Toxicology and Ecology in Ludwigshafen and shipped to Berlin for online-SPE-LC-MS/MS analysis. As previously described 5 μM of rT3 was added to the microsomes therefore it was expected that the sum of measured rT3 and T2 concentrations in the vehicle control (VC) resulted in the initial added concentration (5 μM) because the VC reflected DIO1 activity without any disturbances. In case of inhibitory potential of a substance as well as for the positive control an increase in rT3 concentration and a decrease in T2 was hypothesized. To combine the data from both biological replicates the absolute rT3 and T2 concentrations from each biological replicate were first divided by the given mean concentration of the VC to obtain the ratio TH/VC for every technical replicate. Thus, from the two biological replicates a total of six technical replicates TH/VC ratios were derived per dosage group and per analyte. Except for data from PTU for which four biological replicates were tested and combined for evaluation. Further progression of the plotted data included the transformation to logarithms ($\log_{10}$) of the dosed concentration as well as determination of half-maximal inhibitory concentration (IC_{50}) via nonlinear regression. In Figure 66 concentration-dependent DIO1 inhibition by PTU is simultaneously

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depicted for rT3 and T2 yielding IC_{50} values of $5.76 \mu\text{M}$ and $6.61 \mu\text{M}$ respectively. The previously described hypothesis about the increase in rT3 and the decrease in T2 when DIO1 is inhibited was reflected nicely in both graphs. This was confirmed by the rise in rT3/VC ratio (≥ 1) and the parallel decreasing T2/VC ratio (≤ 1) with increasing PTU concentration.

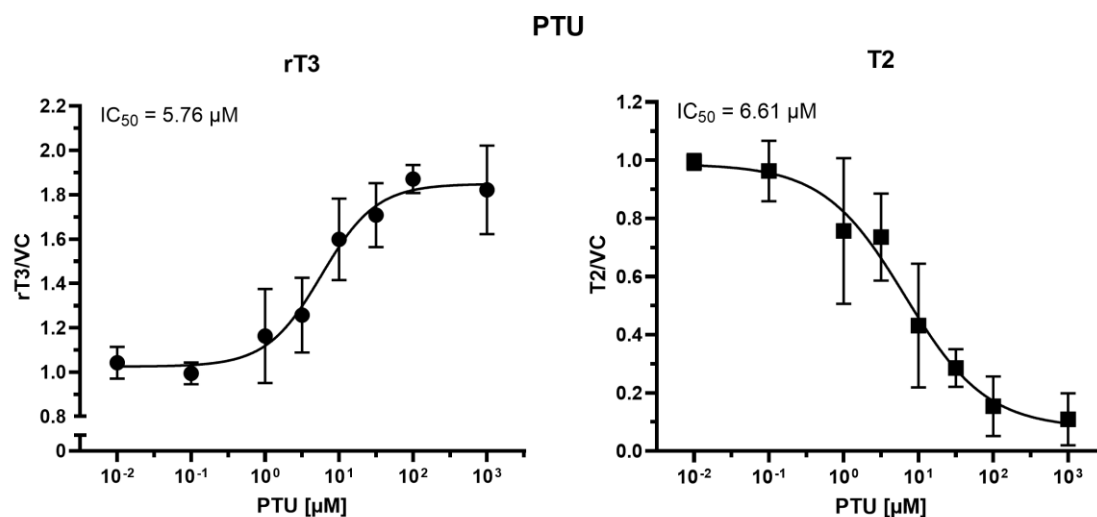


Figure 66. rT3 and T2 concentrations found in microsomes exposed to PTU divided by the mean value of the vehicle control.

The mean data per concentration from four biological replicates were combined and from these an IC_{50} derived. The calculated IC_{50} is listed in each graph whereas rT3 values are shown on the left and T2 on the right which is additionally distinguishable by the symbols showing a dot for rT3 and squares for T2.

This simultaneously occurring inhibition-dependent concentration pattern was not obtained for every substance due to higher fluctuation in the initial added rT3 concentration indicated by overall higher standard deviations compared to T2. For substances 3,3',5,5'-Tetrabromobisphenol A (TBBPA), 3,3',5,5'-Tetraiodothyroacetic acid (TETRAC), Niflumic acid and Amiodarone no rT3 IC_{50} value was derived which could be due to the larger fluctuation in initial added rT3 as seen in Figure 67. For Bisphenol A diglycidyl ether (BPA) rT3 and T2 based calculation of IC_{50} yielded similar results: $18.93 \mu\text{M}$ and $18.74 \mu\text{M}$ as well as for Tannic acid which yielded $7.36 \mu\text{M}$ for rT3 and around $8.81 \mu\text{M}$ for T2. All respective IC_{50} values are summarized in Table 24 which were calculated from the technical replicates ($n=6$) combining two biological replicates which data points are presented in Figure 67 and Figure 68. On contrary to Figure 67, Figure 68 only contains data from substances reflecting no inhibitory DIO1 potential. Tendencies of inhibition were reflected by T2 data (squares) for Ketoconazole, Divanadium pentoxide, and TMTU which were, however, only slightly decreased. Since rT3 did not fluctuate, it was concluded that these substances did not inhibit. For the following substances, no IC_{50} was obtainable: Ketoconazole, Sorbiterome, Divanadium Pentoxide, Resorcinol, and Tetramethyl thiourea (TMTU) which are depicted in Figure 68.

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Overall, T2 results appeared to be more robust as seen in Figure 67 for e.g. TBBPA or Niflumic acid likely because rT3 was added in a surplus and limited capacity of DIO1 enzyme. Therefore, it was concluded that T2 functions as a better readout parameter over rT3 due to being less prone to pipetting errors and due to the conversion capacity of DIO1.

Table 24. Calculated IC₅₀ values [μM] derived from combining results from two biological replicates for each analyte separately.

A slash was added in the given cell when no IC₅₀ value could be derived.

Substance	rT3 IC₅₀ [μM]	T2 IC₅₀ [μM]
PTU	5.76	6.61
Niflumic Acid	/	~ 13.84
Tannic Acid	7.36	~ 8.81
TETRAC	/	3.75
BPA	18.93	18.74
TBBPA	/	8.41
Amiodarone	/	8.05
Desipramine	~ 11.6	29.15
Rosmarinic Acid	~ 101.5	/
Linoleic Acid	~ 100.7	~ 61.26
Silichristin	~ 139.6	/

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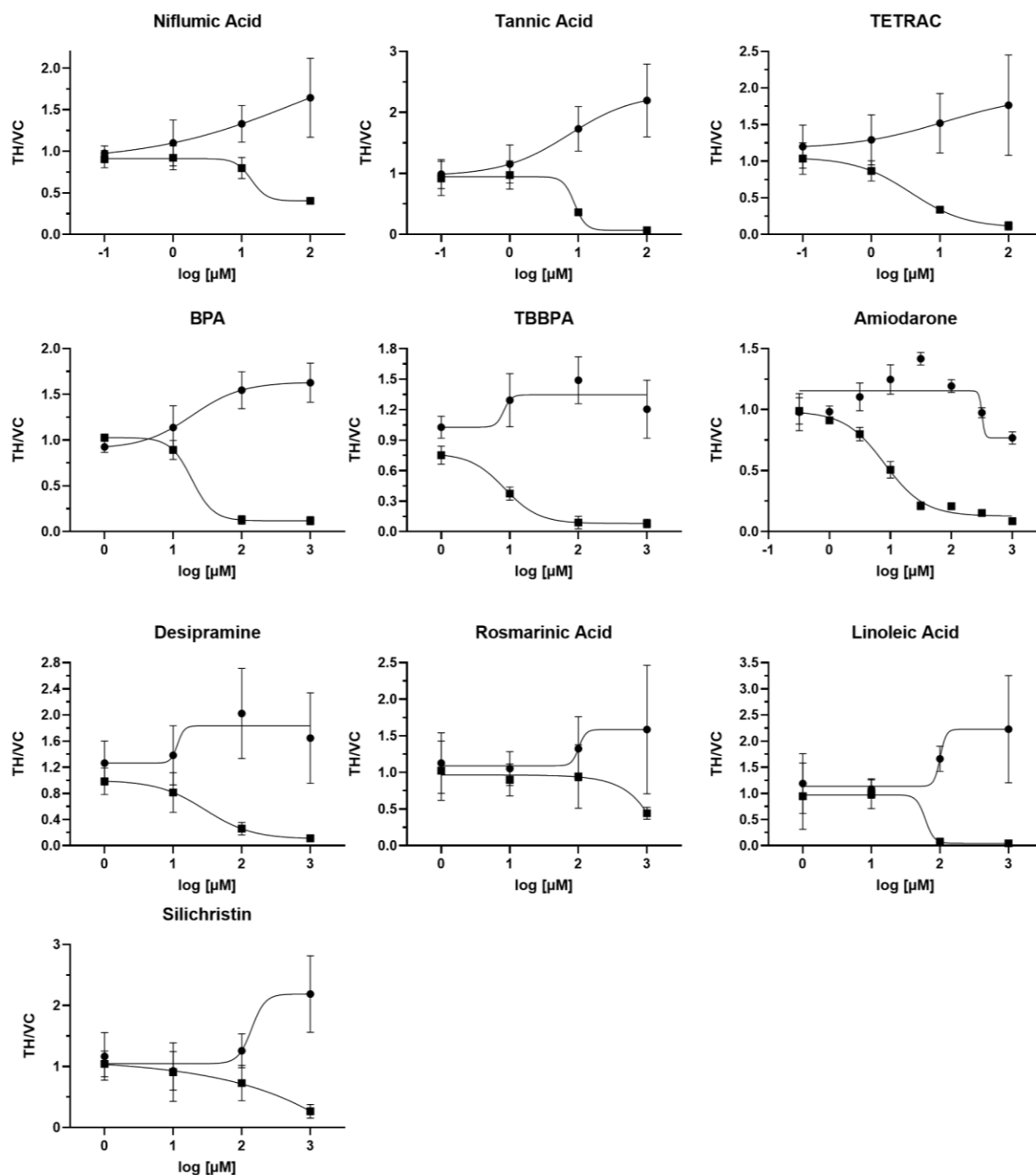


Figure 67. Graphical illustration of rT3 and T2 concentrations in human liver microsomes after exposure to various test substances from which an IC₅₀ value was derivable.

On the y-axis the ratio of rT3 or T2 at a given concentration to the corresponding VC is plotted and the logarithm transformed concentrations of the given substance are shown on the x-axis. Analytes rT3 and T2 are distinguishable by symbols whereas dots refer to rT3 and squares to T2. All graphs contain results from two biological replicates combined.

5.9 Alternative Test Methods to Quantify TH

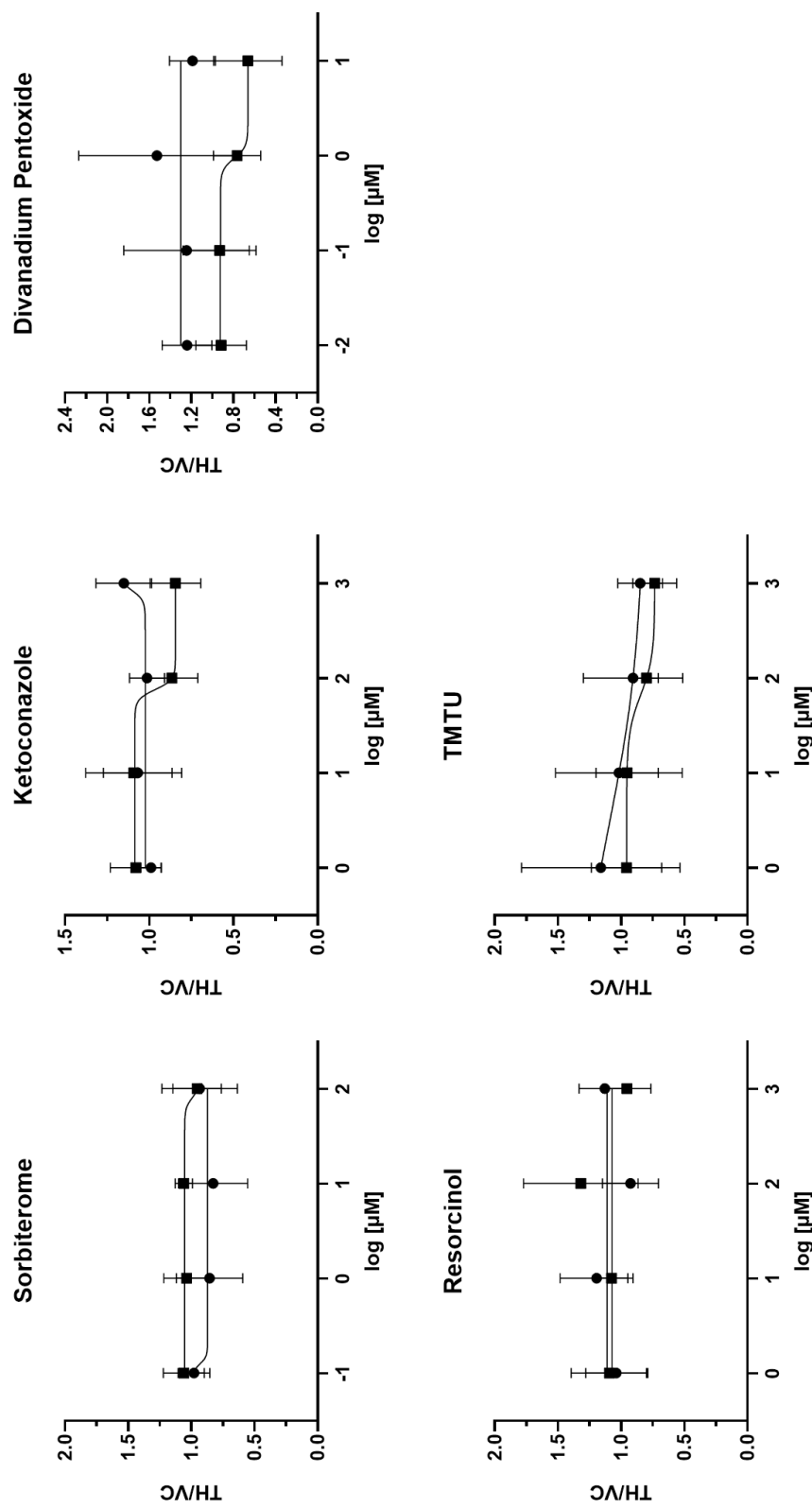


Figure 68. TH/VC ratios obtained from exposure to substances from which no IC_{50} value could be derived. The ratios for rT3 and T2 to the respective VC are indicated on the y-axis and data points are differentiable by symbol: dots rT3 and squares T2. The logarithm transformed concentrations of the given substance are shown on the x-axis. Moreover, the data for each concentration were obtained from two biological replicates. The depicted substances did not demonstrate inhibitory potential of DIO1 in human liver microsomes.

6 Discussion

6.1 Method Development and Detection of Free THs

At the beginning of method development two different approaches were tested regarding their efficiency to analyze free THs. The most commonly used approaches are UF and RED wherefore these were selected. UF procedure was optimized regarding centrifugation time and force as well as it was tested to pre-rinse the membrane with water to improve recovery of free THs. On the contrary, RED procedure was improved by examining various incubation times and rates of mixing. For both approaches volumes of 200 μ L and 300 μ L were evaluated since the goal was to use as less volume as possible to improve applicability for rat studies since plasma volume is limited especially in offspring animals. It was finally decided to proceed with the RED approach incubating 200 μ L plasma samples for 6 h at 300 rpm at 37 °C because it showed more promising results as well as the detected TH concentrations after UF procedure were much lower than using RED.

The main information about method set up and conduction was retrieved from the respective user guides for each device^{143, 144}. Besides that, the optimization of certain parameters as described in a number of different publications had been taken into account when optimizing the used methodology. Such as from Tanoue et al. regarding the decision for a suitable ultrafiltration device. The authors compared three different 30 kDA cut-off devices for their measurement of free THs in bovine serum by examining different centrifugation forces. It was finally decided to proceed with the Centrifree YM-30 UF device at centrifugal force of 1,000-2,000 x g because results showed less variation of fT4 and fT3 concentrations compared to the other UF devices¹³². Similar to the presented developed method in this work, fTH analysis was conducted by LC-MS/MS whereas the ultrafiltrate was spiked with an isotope-labeled mixture after UF, purified on an Oasis HLB cartridge (10 mg, 1 mL), evaporated to dryness and finally dissolved in methanol. The authors further compared equilibrium dialysis (EQD) to UF by analyzing bovine serum and human plasma with each method which resulted in the estimation of similar fTH concentrations with both methods wherefore it was concluded to continue using UF¹³². Prior to the method described by these authors, Soldin et al. developed an isotope dilution tandem mass spectrometry method to detect fTH in human serum/plasma in order to compare UF with EQD as well as with direct fT4 analysis via immunoassay. Soldin et al. utilized 0.05 ng/ml d₂-T4 as an internal standard which was added to the ultrafiltrate and then a total volume of 400 μ L was injected for analysis¹⁴⁵. The UF device Centrifree YM-30 (30,000 MW cut-off) was tested at different temperatures ranging from 4 °C to 40 °C showing the best

6.1 Method Development and Detection of Free THs

correlation at 25 °C to EQD which was conducted at 37 °C claiming that the difference in temperature might be due to the different separation membranes used in UF and EQD. Overall, the authors concluded good correlation between results of UF and EQD ($r = 0.954$, $n = 68$), but poor correlation between tandem MS with immunoassay analysis ($r = 0.459$, $n = 154$)¹⁴⁵. This method was further modified by Gu et al., Soldin et al., and Gounden et al. as seen in Table 25¹⁴⁶⁻¹⁴⁸. Another method comparison between EQD-RIA, fTH immunoassay, to EQD-LC-MS/MS was conducted by Yue et al. Their method procedures entails incubation of a 200 μ L human serum samples for 20 h using dialysis plates from Harvard Apparatus to reach equilibrium after which the dialysate was mixed with an IS working solution prior to LC-MS/MS analysis¹⁴⁹. Results indicated good correlation of immunoassays Vitros ECi FT4 (Ortho Diagnostics) and Modular Analytics E170 with ED-LC-MS/MS for both analytes ($r \geq 0.87$)¹⁴⁹. Similarity in detected fTH concentrations using EQD or UF as described in the literature was not reproduced within this project since fTH results utilizing UF were around 10 times lower than using EQD. That is why further development and testing was proceeded with EQD procedure in this work. One possible explanation might be the use of a swinging-bucket centrifuge instead of the recommended fixed-angle. According to the user guide of the UF device, a fixed-angle centrifuge rotor ensures solvent flow by preventing retained protein to accumulate at the edge of the membrane. In the future, a comparison between the centrifuge rotors should be done to confirm or deny an improvement.

6.1 Method Development and Detection of Free THs

Table 25. Excerpt from literature analyzing fTH in human plasma/serum.

The reported fTH concentrations in the cited literature were unified to the concentration unit [pg/mL] in this table. If certain data was not reported, the respective cell is marked with a “nd” (no data).

Literature	Soldin et al. ¹⁴⁵	Gu et al. ¹⁴⁶	Soldin et al. ¹⁴⁷	Gounden et al. ¹⁴⁸	Tanoue et al. ¹⁵⁰	Yue et al. ¹⁴⁹
Species	Human	Human	Human (children 1 month to 18 years)	Human	Bovine, cat, dog, & Human	Human
Separation Method	UF					EQD
Device Producer	Centrifree YM-30, Millipore					96-well dialysis plate from Harvard Apparatus
Cut off Membrane	30 kDa					5 kDa
Sample Volume [μL]	400	400	400	400	400	200
Temperature [°C]	25	25	25 or 37	25 or 37	37	37
Time	1 h	30 min	30 min	30 min	30 min	20 h
Sample Purification	nd	nd	Online SPE	Online SPE	Offline SPE, Oasis HLB cartridge (10 mg, 1 mL)	Online SPE
LOQ [pM]	nd	nd	nd	nd	nd	1.29
FT4 [pg/mL]	15.2	8-21 (2.5th–97.5th percentile)	13-24 (1–18 years)	14 ± 6	17 ± 1.5	12.8-22.2
FT3 [pg/mL]	nd	0.9-4 (2.5th–97.5th percentile)	1.5-6.0 (1 month – 18 years)	1.9 ± 0.6	3.8 ± 0.4	3.62-6.75

Most literature found using EQD or UF focuses on fTH detection in humans since it is a common clinical outcome parameter unlike in animal studies for which typically total TH is detected and overall plasma/serum volume limited. An extract of the literature analyzing fTH in rat plasma or sera is listed in Table 26. Notable is that most measurements were conducted using RIA or Enzyme-Linked Immunosorbent Assay (ELISA) based approaches. Recently Yin et al. published inter alia fTH analysis in rat offspring using an UF approach which they adapted from Tanoue et al. Analysis was conducted on PND7, 14, 21, and on PND35 showing a tendency of increasing FT3 values with age for males and females¹⁵¹. Analyte FT4 yielded primarily lower concentrations compared to FT4 concentrations detected in adult rats as mentioned in Table 26. A discrepancy in the presented results was in particular notable regarding the step of adding IS with EQD/RED or adding it afterwards to the dialysate. As depicted in Figure 14, and Figure 15 the addition of IS during RED usually led to an IS area loss ranging from 70-90 % compared to the addition of IS afterwards which yielded in similar

6.1 Method Development and Detection of Free THs

peak areas when compared to the directly pipetted standard samples. It was assumed that this IS area loss will result in a similar area loss for the analytes since the overall purpose of adding an IS was to mimic any losses obtained during sample preparation. This was underlined by the different calculated concentrations of fT4 and fT3 when IS was added during or after RED. In the upper graph of Figure 15 this described difference was portrayed whereas calculated concentration of fTHs were around 10 times higher when adding IS during RED than afterwards. The calculated loss was around 90 % which aligned with the IS area loss. Calculated fT4 and fT3 concentrations were around 30 pg/mL and 4 pg/mL respectively when IS was added afterwards to the dialysate, which was in good agreement with the listed literature data in Table 26. In general, it can be said that the addition of IS should adjust for adsorptive losses since the analytes could bind to container walls, filters and membranes or losses due to solvent evaporation¹⁵². Since the isotopically labeled THs are structurally identical to endogenous TH, they were suspected to bind to the plasma TH-binding proteins leading to an unknown concentration of unbound IS¹⁵². Further this would disturb the equilibrium and might lead to false high detected fTH concentrations. In the shown experiment the addition of either all IS or only singular ones such as ¹³C₆-T1 were tested regarding the subsequent calculated concentration of fTH analytes which outcome was displayed in Figure 13. Falsely high calculated recoveries of the analyte concentrations were in particular detected when evaluation of the analytes was examined with their direct IS analogue meaning e.g. T3 with ¹³C₆-T3 (squared bar). However, when T3 was evaluated with a structurally similar IS such as ¹³C₆-rT3 or ¹³C₆-T1 (dark grey and black bar) instead of ¹³C₆-T3, the resulting recovery was similar to the one calculated when the respective IS was added afterwards (light grey bar). Consequently, it was decided that adding a structurally similar IS such as ¹³C₆-rT3 or ¹³C₆-T1 should be suitable to add during RED to compensate for loss during dialysis and the assumption was that the competitive binding towards plasma TH-binding proteins was negligible especially concerning ¹³C₆-T1 since it was rarely detected in rat plasma. Conversely, this also explained that the detected fTH concentrations were calculated higher than in literature or when adding the IS after RED because IS added during RED adjusted for the detected analyte loss during the procedure. Consequently, the ratio between analyte and IS area resulted in a higher value and thus concentration was calculated to be higher.

6.1 Method Development and Detection of Free THs

Table 26. Overview of some literature analyzing fTH in rat plasma/serum (untreated animals).

The reported fTH concentrations in the cited literature were unified to the concentration unit [pg/mL] in this table. If certain data was not reported, the respective cell is marked with a “nd” (no data). Some of the tabularized concentration values were extrapolated from a reported graph and are indicated by the symbol “~”.

Literature	Walker et al. ¹⁵³	Chang et al. ¹⁵⁴	Chang et al. ¹⁵⁵	Hallgren et al. ¹⁵⁶	Martin & Klaassen ⁵⁷	Broniowska et al. ¹⁵⁸	Yin et al. ¹⁵¹
Year	1980	2007	2008	2002	2010	2018	2024
Strain	Sprague-Dawley	Female Sprague Dawley	Male & Female Sprague Dawley	Female Sprague Dawley	Male Sprague Dawley	Male Wistar	Sprague Dawley
Age	PND5-Adult	8-10 w	8-10 w	7 w	Adult	Adult	PND7, 14, 21, & 35
Separation Method	Binding Method	EQD	EQD	Nd	nd	nd	UF
Detection Method	Binding Method	RIA or Chemiluminometric immunoassays	RIA	RIA	RIA	ELISA	ULPC-MS/MS
Device Producer	Sephadex	Nichols Institute Diagnostics Or Bayer ADVIA Centaur® clinical analyzer	Nichols Institute Diagnostics	Johnson and Johnson	nd	DiaMetra	Centrifree YM-30, Millipore
Sample Volume [μL]	nd	nd	nd	Nd	nd	nd	400 (serum)
LOD [pg/mL]	nd	nd	nd	Nd	70 fT4 0.2 fT3	Sensitivity 0.5 fT4 0.05 fT3	14 fT4 7 fT3
FT4 [pg/mL]	946-2800 (PND5-Adult)	18.2-22.1 (range from detection methods)	Females 19.2-26.3	~ 11.7	~ 20	~ 20	PND7-35 Males 100-500 Females 50-100
FT3 [pg/mL]	0.16-2.88 (PND5-Adult)	nd	nd	Nd	~ 3	~ 2	PND7-35 10-20

In conclusion, the developed RED plus online-SPE-LC-MS/MS method is suitable for determining fTHs in rat plasma. However, the detected fTH concentrations were higher than described in cited literature which was due to the addition of IS during instead of after RED which compensated for the detected area loss. The discrepancy between reported concentrations in literature and this work was assumed to be no complication for an evaluation about whether a substance is affecting fTH concentrations since the general effect (increase or decrease) will be still derivable as it will be further discussed in 6.3.4. Furthermore, it was decided to not use the direct IS analogue such as e.g. ¹³C₆-T3 for T3 but instead the structural analogue ¹³C₆-T1 to further prevent influence by possible competitive binding to the plasma TH-binding proteins in

6.2 TH Analysis in Rat Plasma and Brain

plasma. As it seems from the depicted results that $^{13}\text{C}_6\text{-T1}$ less likely binds to the plasma TH-binding proteins than $^{13}\text{C}_6\text{-T3}$ and $^{13}\text{C}_6\text{-T4}$.

6.2 TH Analysis in Rat Plasma and Brain

6.2.1 TH Analysis in Rat Plasma

Total TH analysis in rat plasma has been intensively investigated within the course of this work. Studies were for the most part conducted in Han Wistar rats (CrI:WI(Han)) except for one study which further included Sprague Dawley (CrI:CD(Sprague Dawley)), F-344 (F-344/CrI) and Wistar Han RCC (RccHan:WIST) rats. As outlined in section 5.5 no major difference was detected regarding total TH concentrations between the mentioned rat strains except for T3 and T2 concentrations in male F-344 rats which were above the established range of control Han Wistar concentrations. In Table 27 an overview of exemplary literature measuring total TH concentrations in different rat strains is summarized. The utilized analytical method is also mentioned indicating a majorly use of RIA. Even though the age at analysis differed between the cited literature, the rats can be overall categorized as adults. Within each study in the table, the common difference of total T4 concentration being higher in male rats than in female rats was identifiable. The reported total T4 concentrations in male Sprague-Dawley rats seemed to be higher than in male Wistar rats in studies from Shin et al., Matsuura et al., Martin and Klaassen, and Martin et al. which was not obtained in this work and in reports of Akane et al.^{157, 159-162}. In the last-mentioned study by Akane et al., the detected total T4 values were close to concentrations in Han Wistar rats. The referred increased in total T4 concentration in Sprague-Dawley rats did not necessarily went along with an increase in total T3 concentrations except for data from Matsuura et al. Besides that, total T3 concentrations in both sexes were similar ranging from 0.9 nM to 1.9 nM as seen in Table 27.

The detected results obtained during this work are well comparable to literature independent from the utilized analytical method and from analysis in serum or plasma. This further underlines the robustness and reliability of the developed online-SPE-LC-MS/MS method. Moreover, a good comparability of total TH concentrations between rat strains was perceived.

6.2 TH Analysis in Rat Plasma and Brain

Table 27. Exemplary literature overview of total T4 and T3 concentrations detected in various rat strains.

The literature is sorted by rat strain as seen in the first column which also includes the age at the end of the respective study. If the exact age was not mentioned, the age is referred to as adult. The listed concentrations were unified to the concentration unit [nM] for a better comparison of the data. If no exact value was reported in the given literature, the value was extrapolated from a graph which is indicated by the symbol “~” in front of the value. When no data was available, the given cell is marked with “nd”.

Rat Strain & Age at Analysis	Analytical Method	T4 [nM] Males	T4 [nM] Females	T3 [nM] Males	T3 [nM] Females	Literature
Wistar Adult	RIA (serum)	61 ± 5	nd	1.31± 0.11	nd	Visser et al. 1996 ¹⁶³
SPF Wistar 7 weeks	RIA (serum)	~ 81.1	nd	~1.54	nd	Kawasaki et al. 1998 ¹⁶⁴
Wistar (CrIGlxBrl-Han:WI) 10 weeks	RIA (serum)	48.7 ± 5 49.7 ± 3.3	46.5 ± 9.5 42.6 ± 9.6	1.01± 0.20 0.94± 0.15	1.4±0.26 1.3±0.49	Mellert et al. 2003 ¹⁶⁵
Wistar (HsdCpb:WU) 11 weeks	RIA (plasma)	43.4 ± 10	38.7 ± 7.3	1.07± 0.14	1.05±0.32	Van der Ven et al. 2008 ¹⁶⁶
Wistar (RjHan:WI) 18 weeks	ELISA (plasma)	39.1-66.1 39.9-57.4 51.3-66.4	nd	1.16-1.62 1.18-1.91 1.28-1.86	nd	Wang et al. 2023 ¹⁶⁷
Han Wistar (CrI:WI(Han)) 14 weeks	Online-SPE-LC-MS/MS (plasma)	52.0±6.3 61.1±5.7 59.9±3.6	46.6±11.5 38.4±14.4 44.1±11.3	1.06±0.16 1.15±0.11 1.18±0.07	1.38±0.36 1.12±0.25 1.22±0.21	This Work
Wistar Han RCC (RccHan:WIST) 14 weeks		61.1±9.3 58.1±14.6 64.6±13.6	47.7±15.2 43.5±9.1 51.7±12.4	1.48±0.37 1.21±0.09 1.71±0.43	1.35±0.45 1.30±0.19 1.65±0.39	
Cotton rats (<i>Sigmodon hispidus</i>) live-caught 12-14 weeks	RIA (serum)	~45.05	nd	~1.54	nd	Tomasi et al. 2001 ¹⁶⁸
Sprague-Dawley 9 weeks	RIA (Plasma)	nd	~ 27	nd	nd	Hallgren and Darnerud 2002 ¹⁵⁶
Sprague-Dawley 11 weeks	RIA (serum)	76.7±13.1	38.5±9.0	1.06±0.15	nd	Shin et al. 2006 ¹⁵⁹
Crj:CD(SD)IGS rats (SPF) PND21	RIA (serum)	F0 72.7 ±8.6 F1 72.9 ±8.9	F0 44.9 ± 11.1 F1 50.2 ± 9.0	F0 2.74 ±0.35 F1 2.24 ±0.11	F0 2.61 ±0.44 F1 2.35 ±0.37	Matsuura et al. 2005 ¹⁶⁰
Sprague-Dawley Adult	RIA (serum)	~ 84.95	nd	~ 1.38	nd	Martin and Klaassen 2010 ¹⁵⁷
Sprague-Dawley Adult	RIA (serum)	~ 90.10	nd	~ 1.15	nd	Martin et al. 2012 ¹⁶¹
Sprague-Dawley 9 weeks	Chemiluminescent immunoassay (serum)	43.8±5.2	41.2±10.3	0.56±0.08	0.55±0.10	Akane et al. 2024 ¹⁶²
Sprague-Dawley (CrI:CD(Sprague Dawley)) 14 weeks	Online-SPE-LC-MS/MS (plasma)	48.6±4.8 52.3±7.7 52.8±4.3	36.3±16.4 40.0±7.9 30.6±4.2	1.02±0.13 1.22±0.11 1.33±0.15	1.48±0.19 1.39±0.16 1.22±0.15	This Work

6.2 TH Analysis in Rat Plasma and Brain

Rat Strain & Age at Analysis	Analytical Method	T4 [nM] Males	T4 [nM] Females	T3 [nM] Males	T3 [nM] Females	Literature
Long Evans	LC-MS/MS (serum)	Pooled Pup PND0 ~6.4 PND2 ~12.9 PND6 ~28.3 PND14 ~45.1	GD20 dam ~23.2 Dam PND22 ~54.1	Pooled Pup PND6 ~0.46 PND14 ~0.89	Dam GD20 ~0.81 Dam PND22 ~0.95	Gilbert et al. 2021 ¹⁶⁹
F344/DuCrI 12 weeks	RIA (serum)	~ 64.4	nd	~ 2.30	nd	Chepelev et al. 2017 ¹⁷⁰
F-344 (F-344/Crl) 14 weeks	Online-SPE-LC-MS/MS (plasma)	56.5±7.4 62.1±4.5 66.0±3.9	38.2±7.1 38.9±3.7 41.2±13.6	1.53±0.21 1.92±0.18 1.88±0.18	1.29±0.35 1.35±0.17 1.54±0.47	This Work

6.2.2 TH Analysis in Rat Brain

The following text was copied and adjusted from chapter 4 “Discussion” of the second published paper during this project namely from Hindrichs C, Walk T, Landsiedel R, Kamp H, Schneider S, Melching-Kollmuss S, Funk-Weyer D. Thyroid Hormone Metabolites Quantified in Pup and Adult Rat Cerebellum, Cortex and Whole-Brain Samples Using an Automated Online SPE-LC-MS/MS Method. *Metabolites*. 2024 Jan 17;14(1):61. doi: 10.3390/metabo14010061. PMID: 38248864; PMCID: PMC10820277:

“The robust determination of TH alterations in an offspring brain can become a key parameter in assessing thyroid hormone disruption. This requires in particular a robust and validated analytical method as well as a historical control database of normal, undisturbed TH concentrations in the desired matrix. Based on such a historical control database, it is possible to reliably measure and assess potential alterations on TH concentration after substance exposure. Here, we report on a validated xLC-MS/MS method (online-SPE-LC-MS/MS method) for TH analysis in the brain, yielding LOQs of 0.02 ng/mL for T1, T2 and rT3 and 0.15 ng/mL for T3 and T4. Because of the utilization of a mixed sample preparation, in which we first performed protein precipitation followed by an additional clean-up of the samples via on-line HLB SPE cartridges, it was possible to develop a TH method for the brain matrix with high accuracy and precision. This method was validated by attaining accuracies at LOQ between 90 and 110 % with a corresponding precision of 1.8–9.1 %. Most reports on TH concentration in the brain focus on the analysis of T4 and T3 concentrations. Our method includes additional downstream metabolites, rT3, 3,3'-T2 and 3-T1; of these, rT3 and 3,3'-T2 concentrations were measured in all samples. No difference in TH concentration in the brain was observed between male and female rats at any examined age, which is in line with the

6.2 TH Analysis in Rat Plasma and Brain

available literature^{51, 171, 172}. From PND4 to PND21, an increase in T4, T3, and T2 concentrations and a decrease in rT3 concentration were detected in the brain. TH availability in tissues is closely regulated by deiodinases 2 and 3 during development. It has been shown that in fetal and prenatal rats, DIO3 (inactivating T4 to rT3) expression is high and declines postnatally, whereas DIO2 (activating T4 to T3) expression increases postnatally^{60, 173}. Therefore, we assume that this might be an explanation of decreased rT3 concentrations and increased T4, T3 and T2 concentrations on PND21 in rat brains.

In this study, TH concentrations were further separately analyzed in the cerebellum and cortex. In particular, TH alterations in the cerebellum during fetal and postnatal development are reported to cause intellectual impairments in humans¹⁷⁴. In rodents, TH alterations during the first two postnatal weeks (in this work TH was measured on PND4 and 21/22) could lead to impaired cerebellar function causing impaired motor coordination and balance. In this time, cerebellar morphogenesis such as Purkinje cell dendritogenesis and granule cell migration is affected by THs¹⁷⁵. The investigation of THs in specific brain regions revealed that T4 concentration is similar within age groups in the cerebellum and cortex. T3 and T2 concentrations were lower in the cerebellum on PND4 compared to their values in the cortex, and on PND22, the T2 cerebellum concentration rose to a similar value as in the cortex. The rT3 concentrations followed an opposite pattern, i.e., a similar concentration in both regions on PND4 but a lower concentration in the cerebellum than in the cortex on PND22. When looking at the values in adult rats, there is a high standard deviation in T4 concentration in the cerebellum so that with these data, it cannot clearly be explained whether the T4 cerebellum concentration was higher or similar as in the cortex. The adult rT3 cerebellum concentration as analogous to its concentration on PND22, and the adult T3 concentration in each region was comparable to the value yielded in the cortex on PND4. Similar to the concentration in the cortex on PND4 was the T2 concentration found in the cortex of adult rats, whereas the T2 concentration in the adult cerebellum was slightly higher as in adult cortex.

A comparison of the data reported here to the literature is difficult because of several factors: analysis of different brain regions, efficacy of extraction method, choice of analysis method, examination of different ages, and choice of rat species. One mentioned parameter is the region-specific brain analysis, for which the analysis of either the forebrain or the cortex is mostly found^{50, 87, 103}. Whole-brain analysis is mainly performed when examining fetal pooled brain samples due to the limited quantity of the matrix⁸⁷. Different extraction methods are another critical variable because they *inter alia* have different limitations and handling errors. The liquid–liquid extraction (LLE) method is time-consuming, and large amounts of solvent are

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needed. The method here presented has the advantage that the SPE unit is automated, and therefore, handling errors are negligible. Mixed sample preparation (protein precipitation and online-SPE or LLE and SPE) might be prone to limitations too, since analyte loss could occur over the many procedure steps. However, the stable isotope-labeled internal standard was added early in the sample preparation to intercept analyte loss. Lastly, the analytical method chosen for the readout is influenced by its sensitivity and specificity in detecting the given analytes in a matrix, whereas enzyme-based assays might be more prone to impurities or influenced by the mode of action (MOA) of a substance than LC-MS/MS. A summary of the reported TH concentrations in different brain regions in some of the literature is recorded in Table 28. No data comparable to the here presented method regarding analyzed matrix tissue or age have yet been published to our knowledge.

Table 28. Overview of concentration cited in the published literature.

Analyzed brain region, determined concentration of THs (mainly T4 and T3), and age of rat are listed from each of the literature references.

Age of Rat	Brain Region	TH Concentration	Reference
PND12 males	Half brain	4 ng/g tissue T3	176
PND16 males	Hippocampus and cortex	7 ng/g and 6.2 ng/g tissue T3	177
Fetal GD20	Whole brain	0.6 ng/g T4 and 0.7 ng/g T3	87
PND14	Cortex	3.5 ng/g T4 and 7.9 ng/g T3	87
PND0 and 2	Forebrain	~0.8 ng/g T4 ~1 ng/g T3	172
PND6 and 14	One hemisphere	~1.5 ng/g and ~3 ng/g T4 ~2 ng/g and ~4 ng/g T3	172
PND2	Telencephalon	~0.5 ng/g T4 ~1.6 ng/g T3	51
Adult males	Cerebellum	~0.075 ng/g rT3 ~1500 fmol/g T3 (= 9.76×10^{-10} ng/g) ~3500 fmol/g T4 (= 2.72×10^{-9} ng/g)	178

Additionally, the following table (Table 29) gives an overview of the method performance in the quantification of TH concentrations (LOQs and minimum quantification limits (MQLs)) in the brain reported in the literature. In accordance with the mentioned LOQs in Table 29, the defined LOQs for the brain used in this method were 0.02 ng/mL for T1, T2 and rT3 and 0.15 ng/mL for T3 and T4. In this publication, the reported accuracies ranged from 90 to 110 % with a precision range of 1.8–9.1 %. This is comparable to the published recoveries of 94.9–102 % in brain tissue ¹⁷².

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Table 29. Literature review of MQL and LOQ for TH measurement in rat brains.
Besides MQL and LOQ, the used analytical method and corresponding analyte are listed.

Method	LOQ/MQL	Analytes	Reference
Stable isotope dilution LC-MS/MS	0.005 ng/g	T4 and T3	87, 179
RIA	1.09 ng/mL	T4	177
	6.67 ng/dL	T3	
	0.105 ng/g	T3	
LC-MS/MS	0.04 ng/g	rT3	172
	0.09 ng/g	T4	
LC-MS/MS	0.01 ng/g	T4 and rT3	51
	0.05 ng/g	T3	

Besides the difference in region-specific brain analysis found in the literature, sample preparation reflects a big source of technical variation. The majority of the published methods for sample preparation are originally based on liquid–liquid extraction (LLE) using chloroform and MeOH followed by further purification with Bio-Rad AG 1 × 2 resin columns, as first reported by Morreale de Escobar et al. in 1985 whose method was based on previous descriptions by Folch et al. and Gordon et al.¹⁸⁰⁻¹⁸². Over the years, sample preparation was further optimized by adjusting LLE parameters, using SPE cartridges instead of a resin column and optimizing the washing step during the SPE procedure to further remove phospholipids^{87, 172, 176}. During method development of this work, the described LLE was tested, compared to previously used sample preparations and evaluated regarding measured TH concentration and recovery of a spiked amount (data not shown). No significant improvement using LLE was reached; therefore, sample preparation was finalized using the procedure described in section 4.1.2. Brain pool replicates were included in each analysis to determine the technical variability of the used sample preparation. The derived RSDs were all below 20 %, reflecting the low influence from technical variability of the sample preparation on the final calculated concentration. Therefore, it is assumed that the major influence on sample group variation is thus caused by biological differences between the animals. For a better estimation of technical variability in the future, technical replicates of matrix pool samples should be constantly generated and included in each analysis.”¹⁸³

6.2.3 TH Analysis of Fixed Half-Brain Tissues

Two sample preparation testing procedures were conducted to analyze TH concentrations of FF and PE half rat brain as described in 4.1.3. The results of the analyzed technical replicates indicated mostly the detection of analytes T4 and T3 which were however not consistently detected in every technical replicate. Detected concentrations in general were 12 to 20 times lower than expected in adult rat brain. These observations imply that neither of the testing procedures were sufficient to detect TH concentrations from fixed tissue. Testing procedures

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were adapted from various publications focusing on for example protein or RNA extraction from FF or PE tissue since no publication could be found about TH analysis (status in January 2025)¹⁸⁴⁻¹⁸⁶. FF and PE tissues were first fixed using a 4 % aqueous Formaldehyde solution to harden the tissue and preserve it for long-term storage. It acts by cross-linking proteins with different binding affinities whereas for example tyrosine rings were associated to be of importance¹⁸⁷ which is a component of THs. Assumably testing procedures did not sufficiently weaken the mentioned binding since TH analysis did not yield the expected TH concentrations. Another suggestion is to adjust the MS method accordingly to potential masses of THs bound to Formaldehyde. This step primarily needs direct injection of a prepared FF or PE sample solution into the MS instrument to investigate for potential masses which in a second step if identified, would be needed to integrate into the existing online-SPE-LC-MS/MS method. Other possibilities regarding sample preparation are a longer incubation period of PE tissue in hot water to ensure sufficient removal of paraffin wax or the pulverization of the brain tissue in the presence of liquid nitrogen to in particular improve pulverization for PE samples.

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In section 5.7 the potency of PTU, MMI, ETU, Phenyl Isothiocyanate, Aroclor 1254, Vinclozolin, TBBPA, PFHxS and of various insecticides to alter total TH concentrations in rat plasma was described. These substances can be divided by their MOA which either directly or indirectly influence the TH homeostasis. PTU, MMI, and ETU directly inhibit the TPO enzyme in the thyroid gland which consequently disturbs TH synthesis leading to decreased concentration in plasma. Phenyl Isothiocyanate further directly inhibits TH synthesis by inhibition of NIS while Aroclor 1254 and Vinclozolin indirectly affect TH concentrations via a secondary mechanism evolving from their primary MOA. Vinclozolin and PCBs such as Aroclor 1254 have been shown to be effective in reducing TH concentrations because of UDP-GT activity induction caused by liver enzyme induction in rats^{160, 188}. Rat plasma analysis revealed that the strongest decrease was caused by PTU and MMI, but also the percent decrease of T4 and rT3 concentrations caused by the indirect THD Aroclor 1254 was comparable to PTU and MMI (≥ 90 %). T4 and rT3 concentrations were decreased less strongly by Vinclozolin and ETU than by PTU and MMI (50-70 %). Aroclor 1254, and Vinclozolin administration yielded a similar decrease in T3 concentrations of around 50 % compared to the control. Solely ETU showed a different T3 alteration pattern since its concentration increased. Even though ETU, PTU and MMI share the same MOA, ETU did not change THs to the same extent which might be due to different binding affinities towards TPO. TPO binding affinities have been assessed

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in-vitro by different assays and yielded an IC₅₀ range of around 0.2-10 µM for PTU, of around 0.06-2 µM for MMI, and of around 7.8 – 200 µM for ETU¹⁸⁹⁻¹⁹¹. TBBPA and PFHxS indirectly affect TH homeostasis via a different MOA than Aroclor 1254 and Vinclozolin. These substances competitively bind to plasma TH-binding proteins and displace T4 or T3 from their binding site. Most of the analyzed insecticides are assumed to interact with the TH system through various MOA such as by inducing liver enzymes, binding to plasma TH-binding proteins and/or inhibiting TR.

6.3.1 Direct TH-Related MOAs

When PTU was given to rats at doses of 1 or 10 mg/kg bw/d a dose responsive decrease over most sampling days with up to 90 % decline in total T4, T3 and rT3 concentrations was caused. The same effect was detected for MMI dosed at 25 or 100 mg/kg bw/d. The following discussion about literature reports on PTU, MMI, or ETU exposure are listed in Table 30. Mellert et al. administered three different doses of PTU (0.1, 1, or 10 mg/kg bw/d) for 28 days to male and female Wistar rats (CrI:GlxBrl-Han:WI) and analyzed serum TH concentrations using RIA. The results demonstrated as well a dose responsive effect with reduction at the HD of total T4 by around 77-84 % and of total T3 by 68-73 %¹⁶⁵. Another study by Wang et al. exposed adult rats (RjHan:WI) to PTU by diet containing either 5 ppm or 50 ppm resulting in a calculated daily intake of 0.2 and 2.2 mg/kg/d of PTU. The rats were divided into three subsets with different exposure periods starting from 2 weeks to 4 weeks and up to 4 weeks with a 2-week recovery period and TH concentrations were analyzed via ELISA kits. Reductions of around 53 % for total T3 and around 70 % for total T4 were detected at the HD compared to the control¹⁶⁷. In another study 0.1 % (wt/vol) PTU or MMI (100 mg/kg bw/d) was administered via drinking water to male Wistar rats for two weeks after which serum total T4 and T3 concentrations were measured using RIA. Total T4 and T3 concentrations decreased by around 78 % and 61-64 % compared to control for exposure to each substance¹⁶³. Lastly, Akane et al. administered various concentrations of PTU or MMI ranging from 0.03 to 10 mg/kg bw/d to CrI:CD (Sprague-Dawley) rats by gavage for 28 days from which inter alia TH concentrations were determined using a chemiluminescent immunoassay. PTU dependent T4 and T3 declines were significant at 1 mg/kg bw/d for males and at 3 mg/kg bw/d for female rats reaching a reduction of around 66-68 % for T4 and 41-56 % for T3 at the mentioned concentrations¹⁶². TH alterations caused by MMI were significantly reduced at a dose of 3 mg/kg bw/d in males and only for T4 in females at 10 mg/kg bw/d. At the named doses T4 and T3 were declined by 50 % and 29 % respectively in males and T4 in females by around 69 %¹⁶². All in all, the reported pattern of TH decline in rat plasma caused by PTU or MMI exposure aligned with the presented

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results. Reduced TH concentrations after MMI administration were more evident in the presented results compared to the cited literature, however this might be due to the higher dose (25 or 100 mg/kg bw/d) given. The last TPO inhibitor investigated was ETU which was given via the diet at 75 or 300 ppm (6 or 24 mg/kg bw/d) and revealed a moderate decrease of total T4 but an increase of total T3. Kawasaki et al. applied 0.15 mM/kg or 0.3 mM/kg of ETU (15.3 or 30.6 mg/kg bw/d) for 14 days to male Wistar rats by gavage. Subsequent analysis of total T4 revealed a dose-dependent decrease compared to the control from around 57 % up to 85 % as well as a significant reduction of total T3 concentration at HD of about 50 %¹⁶⁴. Another study by Nouh et al. examined male albino rats that were exposed to ETU (500 ppm = 100 mg/kg bw/d) via the diet for 90 days. Sampling days were on day 7, 30 and 90 revealing a time-dependent decrease of total T4 and T3. Total T3 concentration reduced by around 26 % and total T4 by around 41 % after 90 days¹⁹². These results were in agreement with the detected reduction of total T4 concentration, however striking was the here detected increase of total T3 concentration which was in contrast to reports from the mentioned literature which could be due to the lower doses given to rats examined in this work (6 or 24 mg/kg bw/d compared to 15, 31 or 100 mg/kg bw/d). Since Kawasaki et al. further reported an increase in T3 concentration of around 20 % compared to the control group after exposure to LD (15 mg/kg bw/d) which was not significant.

Table 30. Literature summary of TPO inhibitors affecting total T4 and T3 concentrations in rat plasma.

For the most part studies were done in Wistar rats except for Akane et al. 2024 which was in Sprague-Dawley rats and Nouh et al. 2013 in Albino rats which is further indicated by the symbol “#”. Age ranged from 5 weeks to 16 weeks at the start of the studies representing young adult to adult rats. None of the cited literature utilized online-SPE-LC-MS/MS or LC-MS/MS for TH detection. The symbol “*” refers to a significant alteration. For calculation to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d or a drinking volume of 25 mL/d was assumed, if not stated otherwise in the respective literature.

Substance & Dose [mg/kg bw/d]	Exposure Period [days]	T4 Change [%]		T3 Change [%]		Literature
PTU 100 (drinking water)	14	Males -78.7*		Males -61.1*		Visser et al. 1996 163
MMI 100 (drinking water)		Males -78.7*		Males -64.1*		
PTU 0.1 1 10 (gavage)	28	Males +5.8 to +15.2 -56.5 to -60.8* -83.0 to -84.4*	Females -14.8 to -22.6 -49.5 to -66.7* -77.3 to -78.9*	Males -14.8 to -22.6 -49.5 to -66.7 -77.3 to -78.9*	Females -6.9 to -11.5 +3.8 to -18.8 -68.1 to -71.5*	Mellert et al. 2003 165
PTU 0.2	(A) 14	(A) -38.6*		HD (A)		Wang et al. 2023

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Substance & Dose [mg/kg bw/d]	Exposure Period [days]	T4 Change [%]		T3 Change [%]		Literature
2.2 (diet)	(B) 28 (C) 28 + 14 recovery	-67.4* (B) -41.6* -70.2* (C) -3.5 +22.6*		-53.2* (B) -51.7* (C) -5.1		¹⁶⁷
PTU 0.03 0.1 0.3 1 3 (gavage)	28	Males +44.1* +17.6 -29.4 -67.6* -70.6*	Females +15.6 -18.8 -28.1 -56.3* -65.6*	Males -7.1 -3.6 -7.1 -41.1* -53.6*	Females +5.5 +10.9 +10.9 +9.1 -56.4*	Akane et al. 2024# ¹⁶²
MMI 0.3 1 3 10 (gavage)		Males +14.7 +20.6 -50.0* -67.6*	Females -6.3 +3.1 -28.1 -68.8*	Males +21.4 +7.1 -28.6* -48.2*	Females +10.9 +14.5 +7.3 -25.5*	
ETU 15.3 30.6 (gavage)	14	~ -44.4* ~ -84.1*		~ +20.0 ~ -50.0*		Kawasaki et al. 1998 ¹⁶⁴
ETU 100 (diet)	90	30 days -25.8* 90 days -41.0*		30 d -24.3* 90 d -26.6*		Nouh et al. 2013# ¹⁹²
PTU 1 10 (gavage)	28	Males -89.7* -98.7*	Females -62.0* -97.0*	Males -60.6* -89.8*	Females -13.7* -78.3*	This Work
MMI 25 100 (gavage)	14	Males -93.7* -98.1*	Females -91.5* -96.7*	Males -91.4* -96.0*	Females -82.6* -93.7*	
ETU 6 24 (diet)	28	Males -23.0* -49.2*	Females -21.2 -38.6*	Males +33.5* +32.6*	Females +59.5* +139.3*	

Perchlorates, thiocyanates, or nitrates block the binding site of NIS so that Iodide cannot be transported into the thyroid follicular cell any longer causing decreased TH synthesis. A compound representative for this class is Phenyl Isothiocyanate which was dosed to rats at either 15 or 30 mg/kg bw/d. During the study HD of Phenyl Isothiocyanate was reduced to 22.5 mg/kg bw/d and thus no blood sampling was conducted of the HD group on day 14. The results indicated a stronger decline in males than in females since the adjusted HD after 28 days did not yield a significant change opposed to after 7 days with the initial HD. In a study from 1985 by Speijers et al. Phenyl Isothiocyanate were administered five times per week to SPF-derived male Wistar rats at doses of either 0, 2.5, 10, or 40 mg/kg bw/d for a total of four weeks.

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Total and free T4 were measured in serum using RIA reaching significant reduced concentrations at the highest dose compared to the control (total T4 decreased by around 42 %) ¹⁹³. The significant reduction in T4 concentration was around 42 % which is similar to the calculated T4 reduction in this work of 43 % after 28 days. However, in the course of this study HD was adjusted from 30 to 22.5 mg/kg bw/d which is almost half of the HD administered in the publication by Speijers et al. A reason for this similar reduction even though different doses were applied, might be the difference in duration of administration. Administration in this work was continuous for 28 days whereas Speijer et al. administered the dose for 5 days per week for a total of 4 weeks resulting in 2 days per week of recovery for the rats.

6.3.2 Indirect TH-Related MOAs

As mentioned above, the resulting total T4, and rT3 concentrations in rat plasma after treatment with Aroclor 1254 were similar to the reduction caused by PTU and MMI. This is remarkable since Aroclor 1254 acts primarily by inducing liver enzymes which indirectly affects TH concentration by enhanced TH clearance. This strong effect on TH levels was not determined after e.g. Vinclozolin exposure (as well as other liver enzyme inducers reviewed by Marty et al. ¹⁹⁴) and remains surprising. Discrepancy occurred between the reduction of total T3 concentration which was rather moderate after Aroclor 1254 exposure yielding a decrease ranged between 42-52 % compared to 68-96 % by PTU and MMI. Summary of exemplary literature is enumerated in Table 31 for Aroclor 1254 and Vinclozolin and in Table 32 for TBBPA and PFHxS.

Hallgren and Darnerud exposed female Sprague-Dawleys inter alia to 4 mg/kg bw/d of Aroclor 1254 for 14 days resulting in reduced total T4 concentration by 67 % as well as fT4 by 33 % ¹⁵⁶. Martins et al. reported in two separate publications in male Sprague-Dawleys rats total T4 reductions of ~ 93 % and ~ 95 % as well as for total T3 of ~ 33 % and ~ 53 % at doses of up to 32 mg/kg bw/d ^{157, 161}. The fungicide Vinclozolin was given to Cotton rats (*Sigmodon hispidus*) to determine whether it compromises with their thyroid function. Two doses were chosen based on the approximate exposure rats would encounter in agricultural fields and the second dose was chosen to represent possible bioaccumulation in the fields. Thus, 4 mg/mL or 400 mg/mL were sprayed on rat diet resulting in an approximate daily intake of 6.1 and 610 mg Vinclozolin/kg bw/d which was given for 4 days. Significant reductions in total T4, T3 and free T4 concentrations were detected in the HD groups, but not for free T3 concentration ¹⁶⁸. A 28-day study of Vinclozolin administration via gavage at doses of 0, 3.125, 12.5, 50, or 200 mg/kg bw/d in Sprague-Dawley rats caused a decrease of up to 55 % in females and 73 % in males of total T4 concentrations in the HD group whereas total T3 was not significantly altered ¹⁵⁹.

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Matsuura et al. exposed rats (Crj: CD(SD)IGS rats (SPF)) to Vinclozolin (0, 40, 200 or 1000 ppm) from 10 weeks prior mating until PND21 whereas the F1 generation (offspring) was treated similar as the maternal animals until PND21. During the pre-mating period the doses of the corresponding concentrations were stated to be in females 2.6, 12.8, or 64.0 mg/kg bw/d respectively which was similar to the daily intake in males¹⁶⁰. Significant reductions of T4 as well as T3 concentrations were established in the HD group of the parental animals compared to the control group ranging from 38 to 58 % decline of T4 and 23 to 25 % of T3¹⁶⁰. The same effect was additionally detected in the adult F1 generation except that T3 in females was not significantly altered as well as the percentage of decline was closer between the sexes (T4 reduced by 39 to 45 % and T3 by 14 to 15 %)¹⁶⁰. Overall, this decrease was similar to the detected decrease of 50-70 % for total T4, T3 and rT3 determined with the analytical method developed in this work.

The classes OC (substances G and H) and Phenyl Pyrazole (substance K) have been categorized in the result section 5.7 under subsection 5.7.2.1 which contained results of substances assumed to interact secondarily with the TH system by primarily inducing liver enzymes due to the reason that some representatives have been categorized to cause hypothyroidism through this MOA by EFSA¹⁹⁵. The TH reduction observed in rat plasma after 7-day exposure to substance K yielded comparable percentages to values caused by liver enzyme inducer Vinclozolin as for example T3 was reduced by around 25 % and rT3 by around 33 to 39 %. The T4 reduction caused by Vinclozolin was more profound in males (decline ~ 51 %) than by substance G (decline ~ 31 %) after 14 days, but reduction of T3 was comparable by around 24 to 36 %. In females a similar decline of T3 was detected after 14 days (decline ~ 50-53 %) which was not reproducible for T4 or rT3. On contrary to the other liver enzyme inducing substances, substance H reduced TH concentrations the least strongly and only a significant reduction of T4 in males after 7 days was determined.

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Table 31. Summary of stated literature and this work about TH concentrations in rat plasma after exposure to Aroclor 1254 or Vinclozolin.

For the most part studies rat age ranged from 6 weeks to 10 weeks by beginning of the studies which all can be summarized as adult rats. Furthermore, in each cited literature RIA was utilized for TH detection unlike in this work in which online-SPE-LC-MS/MS was used. When no data was available, it is marked with “nd”. The symbol “*” refers to a significant alteration compared to the control group. For calculation to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d was assumed, if not stated otherwise in the respective literature.

Substance & Dose [mg/kg bw/d]	Exposure Period [days]	Rat Strain	T4 Change [%]	T3 Change [%]	Literature
Aroclor 1254 4 (gavage)	14	Females Sprague-Dawley	-29.6*	nd	Hallgren and Darnerud 2002 ¹⁵⁶
Aroclor 1254 4 8 16 32 (gavage)	7	Males Sprague-Dawley	~ -66.7* ~ -84.8* ~ -92.4* ~ -97.0*	~ -22.2 ~ -33.3 ~ -44.4* ~ -27.8	Martin and Klaassen 2010 ¹⁵⁷
Aroclor 1254 32 (gavage)	7	Males Sprague-Dawley	~ -98.6*	~ -53.3*	Martin et al. 2012 ¹⁶¹
Vinclozolin ~ 6.1 ~ 610 (diet)	PND15 to 90-100	Cotton rats (<i>Sigmodon hispidus</i>)	~ +20.0* ~ -14.3*	~ -10.0* ~ -30.0*	Tomasi et al. 2001 ¹⁶⁸
Vinclozolin 3.125 12.5 50 200 (gavage)	28	Males & Females Sprague-Dawley	Males -17.8 -13.9 -39.6* -73.0* Females -0.3 -21.1 -35.3* -66.2*	Males +0.7 +28.7 -7.9 -18.7 Females nd	Shin et al. 2006 ¹⁵⁹
Vinclozolin ~ 2.6 ~ 12.8 ~ 64.0 (diet)	70 prior mating until PND21	Males & Females Crj: CD(SD)IGS rats (SPF)	F0 Males + 2.1 -9.4 -37.9* Females -2.9 -35.8* -58.5*	F0 Males -13.3 -9.9 -23.0* Females +3.5 -6.5 -25.1*	Matsuura et al. 2005 ¹⁶⁰
Aroclor 1254 5 25 (gavage)	28	Han Wistar	Males -76.8* -93.1* Females -58.0* -87.8*	Males -46.4* -42.1* Females -27.8* -51.8*	This work
Vinclozolin 80 240 (diet)	28	Han Wistar	Males - 25.7* -56.1* Females -32.4 -76.1	Males -2.2 -42.0* Females -8.4 -38.4	

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Substance & Dose [mg/kg bw/d]	Exposure Period [days]	Rat Strain	T4 Change [%]	T3 Change [%]	Literature
Substance G 25 (gavage)	14	Han Wistar	Males -30.6* Females -18.6	Males -35.9* Females -52.9*	
Substance H 5 (gavage)	7	Han Wistar	Males -14.0* Females -20.5	Males +8.3 Females -6.8	
Substance K 32 (diet)	7	Han Wistar	Males -41.9* Females -20.7	Males -25.7* Females -20.0*	

TBBPA and PFHxS, known to primarily interact with TH protein binding, caused a decline in total TH concentrations. PFHxS was administered at doses of either 6 or 20 mg/kg bw/d which is similar to a previously conducted study by Ramhoj et al. administering 5 or 25 mg/kg bw/d. The authors conducted an animal study in pregnant Wistar rats which were exposed to PFHxS from GD7 to PND22 resulting in a reduction in T3 of 84 % compared to controls on PND22 in dams as well as in pups T3 was reduced by 7-16 % on PND22 in the HD group compared to the control respectively¹⁹⁶. The corresponding T4 concentrations were reported to be 60 % and 30 % of control T4 concentrations in HD dams at GD15 and PND22 as well as 55 % of controls at PND16/17 in pups¹⁹⁷. Another study administering PFHxS (50 mg/kg bw/d) from GD6 to PND21 to Long Evans rat showed reduced serum T4 concentration in dams around 32 and 24 % on GD20 and PND22, respectively, as well as T3 concentrations were significant reduced as well¹⁶⁹. The detected reduction of T4 in the presented study was more profound especially in males. T4 concentration in males from the HD group was declined by around 86 %, and T3 by 60 % whereas in females the reduction was less strong but significant. The exact TH-related MOA of PFHxS is not fully elucidated yet, however *in-vitro* it has been show that it binds to plasma TH-binding protein TTR which was also shown for TBBPA^{81, 198}. The second compound TBBPA was administered to Wistar rats (HsdCpb:WU) in a subacute toxicity study for 28 days with doses of either 0, 30, 100, or 300 mg/kg bw/d. The HD of 300 mg/kg bw/d aligned with the LD from the study of this project wherefore these values will be compared. In males total T4 concentrations were significantly reduced by around 27 % and T3 was increased by 14 % and in females a non-significant decline of T4 by 10 % and an incline of T3 by 16 % were reported and determined with a radioimmunoassay¹⁶⁶. The presented results of the LD group after 28 days showed a significant decline of T4 and rT3 by around 54-58 % and of T3 by 31 % in males whereas in females only T4 was significantly lowered by 62 % which overall indicates a stronger decline. A different

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publication reported lowered T4 concentrations by 45 % and 46.3 % in males and by 31.8 % and 28.2 % in females compared to the controls after 33-day exposure to either 300 or 1000 mg/kg bw/d respectively¹⁹⁹.

Table 32. Summary of cited literature and this work about TH concentrations in rat plasma after exposure to TBBPA or PFHxS.

When no data was available, it is marked with “nd”. The symbol “*” refers to a significant alteration compared to the control group as well as the symbol “~” indicates that data were extrapolated from a graph. For calculation to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d was assumed, if not stated otherwise in the respective literature.

Substance & Dose [mg/kg bw/d]	Exposure Period	Rat Strain	T4 Change [%]	T3 Change [%]	Literature
PFHxS 25 45 (gavage)	GD7- PND22	Wistar (HanTac: WH)	Dam GD15 LD -40.0* Dam PND22 -52.4* -64.3* Pup PND16 -42.9* -50.0*	nd	Ramhoj et al. 2018 ¹⁹⁷
PFHxS 0.05 5 25 (gavage)	GD7- PND22	Wistar (HanTac: WH)	See above	Pup PND16/17 ~ + 3.0 ~ -10.0 ~ -20.0* Dam PND22 ~ 0.0 ~ 0.0 ~ -20.0*	Ramhoj et al. 2020 ¹⁹⁶
PFHxS 50 (gavage)	GD6- PND21	Long Evans	Dam GD20 ~ -33.3* Dam PND22 ~ -23.8* Pup PND0 ~ -80.0* Pup PND2 ~ -70.0* Pup PND6 ~ -63.6* Pup PND14 ~ -54.3*	Dam GD20 ~ -24.5* Dam PND22 ~ - 16.1* Pup PND6 ~ -43.3* Pup PND14 ~ -32.8*	Gilbert et al. 2021 ¹⁶⁹
TBBPA 30 100 300 (diet)	28 days	Wistar (HsdCpb:WU)	Males -5.5 -3.2 -27.2* Females +3.1 -6.2 -10.1	Males +1.9 +2.8 +14.0* Females +1.9 +14.3 +16.2	Van der Ven et al. 2008 ¹⁶⁶
TBBPA 100 300 1000	13 weeks	CD®(CrI: CD (SD) IGS BR)	33 days Males -26.2* -31.0*	nd	Osimitz et al. 2016 ¹⁹⁹

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Substance & Dose [mg/kg bw/d]	Exposure Period	Rat Strain	T4 Change [%]	T3 Change [%]	Literature
(gavage)			-31.7* Females -22.5* -24.1* -22.0*		
TBBPA 300 1000 (gavage)	28 days	Han Wistar	Males -53.7* -63.7* Females -62.3* -74.3*	Males -31.1* -38.2* Females -23.5 -30.1	This work
PFHxS 6 20 (gavage)	28 days	Han Wistar	Males -84.9* -86.0* Females -42.9* -49.7*	Males -57.5* -59.6* Females -20.0* -13.3	

All in all, the here presented results in sections 6.3.1 and 6.3.2 were in accordance with reports from literature except for a few cases such as T3 concentration after ETU exposure. As for example total T3 concentration after ETU exposure was found to be increased here and by Kawasaki et al. unlike other reports stating reduction of total T3 which studies further administered a higher dose of ETU. ETU seemed to be less potent than PTU or MMI to alter TH concentrations in plasma. The liver enzyme inducer Aroclor 1254 was as efficient as PTU and MMI to reduce total T4 and rT3 concentrations, but not as efficient for total T3 which was reduced by around 50 % instead of around 90 % by the HD of PTU or MMI. The conversion of the HD of Aroclor 1254 and PTU to the concentration in [$\mu\text{mol/L}$] were comparable yielding 76.6 and 58.7 $\mu\text{mol/L}$ respectively but MMI was remarkable higher yielding 875.9 $\mu\text{mol/L}$. This reduction pattern had been found to be reported similarly in literature. Lastly, Vinclozolin overall reduced total T4, T3 and rT3 concentrations in a range between 50-70 % also coherent with other reports. A similar pattern as Vinclozolin was obtained after exposure to substance G and K. The detected TH alterations by TBBPA and PFHxS were found to be more profound using the developed methodology from this work than in the stated publications.

6.3.3 Miscellaneous TH-Related MOAs

Besides the above-mentioned substances, various representative substances from different insecticide classes that are assumed to interfere with the TH system through miscellaneous MOAs have been investigated regarding their effect on TH concentrations in rat plasma and whole brain. Some of the suspected TH-related MOAs are inhibition of TH synthesis, binding to its thyroid hormone receptor (TR), competitive binding to transport proteins in blood or the induction of TH metabolism⁶⁸. These MOAs could possibly cause faster clearance of THs

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leading to lower TH concentration in blood or the interaction with the TR in brain for example might cause disturbance in the development of the CNS which is especially of importance considering fetal and post-natal development⁶⁸. In this work TH concentrations were determined retrospectively in plasma and brain from adult rats from various studies involving pesticide exposure. Pesticides tested were all from different insecticide classes namely OCs, OPs, Pyrethroids, Organotin compounds and Phenyl pyrazoles for either 7 and/or 28 days. TH plasma data of OC and Phenyl pyrazole are discussed in 6.3.2. Every pesticide investigated showed at least one significant effect for one of the TH analytes in one matrix. Usually, the effect was more pronounced in adult rat plasma than in the respective adult rat brains. It is however to be mentioned, that the animals from which the brains had been dissected were only exposed to substances during adulthood, and not during development.

Pyrethroids function by inhibiting voltage-gated Sodium channels in axonal membranes leading to paralysis of the organism. For several pyrethroids as well as for one of their common metabolite which is 3-phenoxybenzoic acid (3-PBA) an antagonistic activity towards TR β has been detected *in-vitro* which might be due to their structural similarity to THs²⁰⁰. *In-vivo* analysis from this work of THs in rat plasma after exposure to substance C indicated reduced T4 concentration but rather increased T3, rT3, and T2 concentrations whereas substance B exposure caused a similar pattern meaning decreased T4 concentration, but increased T3, rT3 and T2 concentrations in this work. In this work TH concentrations detected in rat brain were unchanged except for significantly increased T4, and rT3 concentrations caused by substance B administration and significant decline in rT3 concentration in males after 28-day exposure to substance C. The last tested compound substance A did not lead to significant changes in TH concentrations in neither plasma nor brain from adult rats after 7 days. Unlike all other tested substances T3, rT3 and T2 concentrations increased after pyrethroid exposure. Controversial results in rats were reported as either increase or decrease of TH concentrations after pyrethroid exposure²⁰¹⁻²⁰⁴.

For several organotin compounds interferences in the TH system of amphibians (*Xenopus laevis* tadpoles) and zebrafish have been reported as e.g. alterations of TH concentrations or the regulation of gene expression such as down-regulation of TR β or DIO2²⁰⁵⁻²⁰⁷. Furthermore, Santos-Silva et al. reviewed the TH-related effect of organotin compounds found in literature including for example reduced TH concentrations in rodents but also interaction with TR β expression *in-vitro*²⁰⁸. The organotin compounds tested during this work also revealed reduced TH concentrations in rat plasma. Substance I only significantly reduced T4 by around 35 to

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52 % but increased rT3 concentrations in females. The second substance J caused an equal decline of around 20 to 31 % in T4, T3, and rT3 concentrations after 7 days.

The last insecticide class was OPs which possible TH-related MOAs are not clarified *in-vivo*⁷⁵. Studies in male goldfish (*Carassius auratus*) exposed to different OPs obtained decreased total T3, free T3 and free T4 but unchanged total T4 concentrations as well as altered gene expression in tissues as for example DIOs in various tissues and hepatic TTR expression was transiently induced^{209, 210}. Investigation on *Xenopus laevis* metamorphosis after OP exposure revealed that TH concentrations were altered and the gene expression profile indicated an induction of TTR, DIO2 and TR β ²¹¹. Within this work OPs did not significantly alter T4 or T3 concentrations in plasma. In adult rat brain substance F significantly increased T4, T3, rT3 concentrations as well as T3 was increased in both sexes and T4 in females after exposure to substance E.

Besides TH detection in rat plasma, TH concentrations in adult whole brain were measured as well. Statistically significant alterations in adult rat brain were only detected after exposure to substances B, F, G, H, I, J, and K whereas not for all THs and sex as for example in some cases only one analyte in one sex was significantly altered. On the contrary in rat plasma the TH alteration pattern was typically detectable within more than one TH concentration but still dependent on sex. For most of the substances plasma and brain samples were available after 7-day exposure which is a short period because e.g. dose range studies are typically at least 28 days long. However, for three substances (C, G, and I) samples after 7 days and 28 days were obtainable which was useful to underline possible substance-, and time-dependent effects. Dot plots were generated by contrasting plasma to the respective brain value so that possible time-dependency are made graphically visual. A good indicator for this was the time-dependent change of T3 after substance C exposure after 7 days compared to 28 days in which T3 brain concentration were higher after 28 days in control and dose group. On contrary, T4 and rT3 concentrations were within a similar range in rat brain, but dose group was separated from the control group in rat plasma after both exposure periods. Time-dependent changes in T3 and T2 were also visible for exposure to substance I for which after 28 days T3 and T2 concentrations were decreased in rat brain which effect was not that profound after 7 days. T4 and rT3 concentrations were already lowered after 7 days in rat brain. The last substance obtaining data after both exposure periods was substance G. Due to the low sample set in the HD of males after 28 days (n=2), a conclusion about its effect in plasma and contrasted to brain were not derivable. However, in male rat brains a significant decrease was detected after 28 days for T4, T3, and rT3 as well as T4, T3, and T2 concentrations were significantly lowered in the HD in

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plasma females. Thus, it could be assumed that this is ongoing in plasma of male rats after 28 days additionally because male rats showed decreased T4 and T3 after 14 days. In rat brain no significant change by substance G was detected after 7 days for none of the analytes.

All in all, the investigated insecticides are suspected to interact with the TH system through various possible MOAs leading to mostly decreased TH concentrations in plasma which have been postulated by *in-vitro* or *in-vivo* experiments as explained above. The TH data generated during this work in general aligned with reports from literature which were not collected substance-specific due to the blinding of the substances. The data indicated that the substances to some extent alter TH concentrations in the investigated matrices. However, most of OPs, Pyrethroids and Organotin compounds were not classified as pesticides causing effects on the thyroid by EFSA (when evaluating the compounds for establishing cumulative assessment groups for the thyroid)¹⁹⁵.

6.3.4 Free and Total TH Concentrations after PTU or Aroclor 1254 Exposure

Plasma samples from PTU and Aroclor 1254 studies were additionally examined for their free TH concentration in plasma. The results revealed a similar strong decrease for free T4 after exposure to both substances, but PTU further caused a strong inhibition in free T3 values which was not the same for Aroclor 1254. Aroclor 1254 mostly did not significantly alter fT3 concentration. Except for day 7 in males which led to a significant increase in fT3 concentration compared to control. However, the control group on this day consisted of only three values which might not have sufficiently represented the biological TH fluctuation in this group. Male control groups on day 14 and 28 yielded coherent values (n=5), so that their comparison to HD on day 7 will likely neglect the detected significant increase. Aroclor 1254 and PTU were both administered by gavage and HD was 25 mg/kg bw/d or 10 mg/kg bw/d respectively. Total T4 and T3 concentrations were lowered by up to 90 % after exposure to HD of PTU and Aroclor 1254 however the last substance caused a decline of around 50 % for total T3 concentration in HD group compared to the control group. Only female plasma samples from the study involving PTU were analyzed regarding their free TH concentrations since the volume in male plasma samples was not enough. Free T4 and T3 were both significantly decreased compared to the control by around 90 % for fT4 and around 60-80 % for fT3. Aroclor 1254 exposure led to a decline of fT4 by around 70 % in females and males. On contrary to PTU, Aroclor 1254 did not significantly alter fTH concentrations to the same extent.

The following discussion of literature is additionally tabularized in Table 33. Morse et al. administered Aroclor 1254 to pregnant Wistar WU rats and determined decrease of total and

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free TH concentrations using a chemiluminescence kit²¹². Exposure period was from GD10 to GD16 giving either 0, 5 or 25 mg/kg bw/d and TH analysis was conducted on GD20, PND4, PND21 as well as on PND90. The HD was the same dose as the HD used in the presented study. Dams, fetus and PND4 pups showed the most significant changes in total T4, T3 as well as free T4 concentrations²¹². With increasing age of the offspring, the TH concentrations in plasma seemed to normalize. Dams on GD20 had a loss of around 50 % total T4, 26 % total T3, and of around 58 % free T4 compared to control. Fetal total T4 concentration decreased by 74 %, and free T4 by 81 %. On PND4 total T4, free T4, and total T3 decreased by 46 %, 11 %, 53 % for males and around 64 %, 50 % and 35 % for females, respectively. In a previous study from Goldey et al. who described the effect of inter alia Aroclor 1254 on development, TH concentrations, and auditory function. In their experiment pregnant Long-Evans rats were exposed to Aroclor 1254 by gavage of either 0, 1, 4, 10, or 40 mg/kg from GD6 to PND21. These doses were adapted to 0, 1, 4, or 8 mg/kg in a second cohort because of a pup mortality $\geq 50\%$ ⁷⁷. Total T4 and T3 serum analysis was conducted in offspring on PND1, 7, 14, 21, and up to 45. The HD of 10 mg/kg caused a decrease of total T4 serum by 63 % on PND1, 90 % on PND7, 91 % on PND14 compared to the control. Total T3 concentration was decreased on PND1 by 65 %, on PND7 increased by 11 %, and on PND14 decreased by 21 % compared to the control group. On PND21 no concentration could be measured due to an insufficient number of animals. Moreover, the authors plotted free TH and total TH concentrations in Figure 3 of the cited publication from PND7 until PND 45 for administration of 0, 1, 4, or 8 mg/kg Aroclor 1254⁷⁷. Free T4 showed a similar concentration pattern over this timeline as total T4 with significant decreased values during the first period of development for the two highest doses. On contrary free T3 concentration seemed to be more significantly affected than total T3 in particular on PND21 and PND28 which appeared to be a decline around 50 %⁷⁷. A different study in male Sprague-Dawley rats by Martin and Klaassen which measured free and total T4 and T3 concentrations after gavage of 0, 4, 8, 16, or 32 mg/kg/day Aroclor 1254 for 7 days. The authors reported a reduction of total T4 at the highest dose by around 93 %, free T4 by around 97 %, total T3 by around 30 % which was not significant, and lastly free T3 by around 70 % compared to the respective control¹⁵⁷. A dose-response reduction was detected for total T4, free T4 and free T3¹⁵⁷. Like the previously mentioned publications, a more profound reduction was detected for free T3 concentration than for total T3. Exposure to 4 mg/kg bw per day in female Sprague-Dawley rats for 14 days led to reduced total T4 concentration by $\sim 67\%$ as well as fT4 by $\sim 33\%$ ¹⁵⁶.

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Table 33. Exemplary literature assessing free THs in rats after exposure to Aroclor 1254.

Values that were extrapolated from a graph are marked with a “~” in front of the value. Significant alterations are highlighted with a star symbol “*” and when no data was available, the given cell is marked with a “nd”. For calculation to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d was assumed, if not stated otherwise in the respective literature. If the exact age was not mentioned, the age is referred to as adult.

Parameter/ Literature	Goldey et al. 1995 ⁷⁷	Morse et al. 1996 ²¹²	Hallgren and Darnerud 2002 ¹⁵⁶	Martin and Klaassen 2010 ¹⁵⁷	This Work	
Rat Species & Age at Beginning of Study	Long-Evans (GD2)	Wistar WU 14 weeks	Female Sprague-Dawley 7 weeks	Male Sprague-Dawley Adult	Han Wistar 10 weeks	
Dose [mg/kg bw/d]	1 4 8	25	4	4 8 16 32	25	
Duration	GD6 to PND21	GD10 to GD16	14 days	7 days	28 days	
Method	RIA	Chemiluminescence kit	RIA	RIA	Online-SPE-LC-MS/MS	
Matrix	Serum	Plasma	Plasma	Serum	Plasma	
Total T4 Change [%]	<u>PND7</u> -79.8* -90.5* <u>PND21</u> -87.2* NC	<u>GD20</u> -74.1* Males <u>PND4</u> -46.6* <u>PND21</u> -36.4* Females <u>PND4</u> -64.2* <u>PND21</u> -32.0*	-37.0*	-66.7* -84.8* -90.9* -97.0*	Males <u>7 d</u> -85.8* <u>14 d</u> -91.4* <u>28 d</u> -93.1*	Females <u>7 d</u> -75.6* <u>14 d</u> -87.4* <u>28 d</u> -87.9*
Total T3 Change [%]	<u>PND7</u> -12.0 +11.3 <u>PND21</u> -17.8* NC	Males <u>PND4</u> -53.1* <u>PND21</u> -18.0* Females <u>PND4</u> -35.7* <u>PND21</u> -15.5*	nd	-22.2 -33.3 -44.4* -27.8	Males <u>7 d</u> -35.7 <u>14 d</u> -48.5* <u>28 d</u> -41.7*	Females <u>7 d</u> -33.7 <u>14 d</u> -42.4* <u>28 d</u> -51.9*
Free T4 Change [%]	<u>PND7</u> -60.0* > - 60.0* <u>PND21</u> -83.3* -91.7*	<u>GD20</u> -81.1* Males <u>PND4</u> -11.4 <u>PND21</u> -19.0 Females <u>PND4</u> -50.7 <u>PND21</u> -25.5*	-66.7*	-75.0* -85.0* -92.5* -95.0*	Males <u>7 d</u> -44.4 <u>14 d</u> -72.7* <u>28 d</u> -76.5*	Females <u>7 d</u> -50.8* <u>14 d</u> -80.3* <u>28 d</u> -75.7*
Free T3 Change [%]	<u>PND7</u> N/A <u>PND21</u>	nd	nd	- 49 to 70 %	Males <u>7 d</u> +63.4*	Females <u>7 d</u> -3.6

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Parameter/ Literature	Goldey et al. 1995 ⁷⁷	Morse et al. 1996 ²¹²	Hallgren and Darnerud 2002 ¹⁵⁶	Martin and Klaassen 2010 ¹⁵⁷	This Work	
	-33.3* -66.7*				<u>14 d</u> +15.8	<u>14 d</u> -38.4*
					<u>28 d</u> +34.1	<u>28 d</u> -20.0

PTU doses of 0.01 % or 0.2 % in drinking water (10 or 200 mg/kg bw/d) were given to pregnant Long-Evans until PND14 and free THs were analyzed in serum 6 weeks before breeding, after delivery, or 6 weeks after delivery in dams as well as on PND14 in pups²¹³. In dams, significant declines for both dosages were detected causing a fT4 decline of around 68 % and 87 % and a fT3 decline of around 46 % and 69 %, respectively²¹³. Subsequently in pups on PND14 a similar decline was reported showing a reduction of around 69 % and 91 % for fT4 and of around 52 % and 70 % for fT3²¹³. A similar decline was detected in another study in dams on GD19 and offspring on PND21 of Wistar rats (Harlan, CPB) of which the dams were exposed to 5 mg /L PTU in drinking water (0.5 mg/kg bw/d) from GD7 to PND21 yielding fT4 reduction of around 68 % in dams and offspring, fT3 reduction of around 22 % in dams, and of around 32 % in offspring compared to the respective controls determined via RIA²¹⁴. In the presented work, the HD of PTU yielded 10 mg/kg bw/d which caused a decline of fT4 in adult female rats of around ≥ 90 % and of fT3 around 60 % to 82 %. Especially regarding fT4, the detected effect was slightly more pronounced than in the reported literature as well as fT3 yielded a similar decline of around 68 % to literature after 28 days but reached 82 % after 14 days. In Table 34 the described data are enumerated to obtain a better overview.

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Table 34. Summary of literature containing analysis of fTH in rats that were exposed to PTU.

Significant alterations are indicated by the symbol “*” and “nd” refers to when “no data” was available for the given parameter. For calculation to mg/kg bw/d, a body weight of 250 g and a food consumption of 20 g/d or a drinking volume of 25 mL/d was assumed, if not stated otherwise in the respective literature.

Parameter/Literature	Lilienthal et al. 1997 ²¹⁴	Brosvic et al. 2002 ²¹³	This Work
Rat Species & Age	Time-mated Wistar (Harlan, CPB)	Long-Evans Dams 18 months	Han Wistar 10 weeks
Dose [mg/kg bw/d]	0.5	10 200	10
Duration	GD7 to PND21	Pregnant until PND14	28 days
Method	RIA	nd	Online-SPE-LC-MS/MS
Matrix	Serum	Serum	Plasma
Total T4 Change [%]	<u>Dams GD19 (n=3)</u> -75.4* <u>Pup PND21 (n=8)</u> -72.4*	nd	Females <u>7 d</u> -18.0 <u>14 d</u> -97.7* <u>28 d</u> -97.0*
Total T3 Change [%]	<u>Dams GD19</u> ± 0.0 <u>Pup PND21</u> +10.0	nd	Females <u>7 d</u> -38.6 <u>14 d</u> -84.9* <u>28 d</u> -78.3*
Free T4 Change [%]	<u>Dams GD19</u> -68.1* <u>Pup PND21</u> -68.4*	<u>Dams</u> -66.7* -87.2* <u>Pups PND14</u> -68.8* -84.4*	Females <u>7 d</u> -38.2 <u>14 d</u> -96.8* <u>28 d</u> -94.9*
Free T3 Change [%]	<u>Dams GD19</u> -22.2* <u>Pup PND21</u> -32.1*	<u>Dams</u> -47.1* -70.6* <u>Pups PND14</u> -40.0* -72.0*	Females <u>7 d</u> -59.6* <u>14 d</u> -82.2* <u>28 d</u> -69.0*

The presented published data in comparison with the here presented results were reproducible regarding the sharp decrease in total and free T4 concentration at the highest dose of 25 mg/kg bw/d Aroclor 1254 as well as the moderate decrease of total T3 concentration. However, the stronger reduction in free T3 rather than in total T3 as reported in literature was not detected. Detection of fTH after PTU exposure to adult female rats yielded similar declines as in literature, however it should be considered that the values compared were derived from rats at different life stages. Conclusively, the developed method to detect free TH was successful, however high volume (200 µL) per plasma sample is needed for analysis which is in particular difficult to retrieve from offspring animals. Thus, it is more likely that fTHs will be assessed for substances which are assumed to have specific THD properties (e.g. interact with TH

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binding proteins) and not routinely. A similar alteration of free and total TH concentrations was established for the TPO inhibitor PTU and the liver enzyme inducer Aroclor 1254. In the future substances acting through different TH-related MOAs such as e.g. interfering with the plasma TH-binding proteins such as TBBPA or PFHxS should be analyzed regarding their free TH fraction and compare the detected effect to total TH analysis already conducted in this work. This further investigation will contribute to evaluate the assumption whether alterations of free and total TH concentrations are equally affected in rats and whether the analysis of free THs is a necessary diagnostic parameter in rat studies.

6.4 Non-Supplementation of Iodide in Rat Diet and Its Effect on TH Concentrations

In an animal study conducted to assess the effects of non-supplementation of dietary Iodide on the offsprings TH concentrations in brain and plasma as well as their neurodevelopment, parental rats were given either non-supplemented (NS) Iodide diet or supplemented (S) diet namely the standardized rat diet (control).

Parental animals were exposed to these diets for four weeks prior to mating and the dams further until PND21. Plasma samples were taken from the parental animals after day 7, 14 and 28 for metabolome analysis as well as for the determination of TH concentrations. This was further expanded to TH plasma analysis in the offspring on PND4 and PND21 from which TH concentrations were examined in brain additionally. The Iodide content in the NS diet was around 0.16 mg/kg which is within the dietary requirement for rats of 150 to 200 $\mu\text{g}/\text{kg}$ ²¹⁵, thus a decrease in TH concentrations was not necessarily expected. However, the standardized rat diet which was referred to as supplemented diet yielded an Iodide content of 1 mg/kg which was around six times higher than the NS diet. For the calculation of Iodide intake per day [$\mu\text{g}/\text{d}$] considering the food consumption of the rat, an average food consumption of 20 g/d was considered which was derived from the data obtained during the study. This resulted in an Iodide intake per day for animals receiving S diet of 20 $\mu\text{g}/\text{d}$ and for NS diet of 3.2 $\mu\text{g}/\text{d}$. First, the presented results of plasma TH analysis of the parental animals before mating revealed no changes in total TH concentrations in plasma except for a significant decrease of rT3 in males at day 7 which was not visible anymore at later time points. Table 35 summarizes an extraction of literature studying the effect of Iodide deficient diet in adult rats. Results from this work of parental animals before mating and after 28-day exposure to the respective diets are tabularized with results in adult rats from literature in Table 35. The NS diet (3.2 $\mu\text{g}/\text{d}$) was comparable to the Iodide content in dosage group 2 from Gilbert et al. (4.14 $\mu\text{g}/\text{d}$). Comparing the effect of

6.4 Non-Supplementation of Iodide in Rat Diet and Its Effect on TH Concentrations

these diets revealed no significant changes in T4 or T3 concentrations, but a slight increase compared to the control was observed.

Table 35. Extraction of literature administering ID diet to rats without mating or TH analysis in offspring. “nd” was written in a cell when “no data” was available. The symbol “*” refers to reported statistical significance compared to the respective control. For calculation of Iodide intake per day of the rat, a food consumption of 20 g/d was assumed if not reported otherwise in literature.

Parameter/Literature	Janssen et al. 1994 ²¹⁶	Pedraza et al. 2006 ¹⁰⁴	Gilbert et al. 2011 ¹³⁵	This Work
Rat Species	Male Wistar Rats	Female Wistar	Female Long-Evans	Han Wistar
Age at Beginning of Study	6 weeks	7 weeks	6 weeks	10 weeks
Iodide Intake [µg I/d]	<i>ad libitum</i> C: 3.05 Restricted C-R: 60 % C ID: No KIO ₃	C=LID+5.0: 5.0 LID+1.0: 1.0 LID+0.5: 0.5 LID: 0.052 LID': 0.021	C: 26.02 ± 1.92 DG2: 4.15 ± 0.08 DG3: 2.52 ± 0.04 DG4: 0.65 ± 0.02 DG5: 0.21 ± 0.01	S: 20 NS: 3.2
Duration	6 weeks	12 weeks	8 weeks	4 weeks
Method	RIA	RIA	RIA	Online-SPE-LC-MS/MS
Matrix	Plasma	Plasma	Serum	Plasma
T4 [nM]	C <i>ad libitum</i>: 81 ± 9 C-R: 63 ± 10 ID: 49 ± 8*	C: 29.48 ± 5.02 % of C LID+1.0: ~ 90 LID+0.5: ~50* LID: ~25* LID': 5 %*	C: ~ 48.91 DG2: ~ 57.92 DG3: ~ 48.91 DG4: ~ 38.62* DG5: ~ 25.74*	Males S: 50.77±10.5 NS: 57.74±6.8 Females S: 35.87±11.6 NS: 45.84±14.1
T3 [nM]	C <i>ad libitum</i>: 0.5 ± 0.09 C-R: 0.35 ± 0.09 ID: 0.38 ± 0.15	C: 0.48 ± 0.031 % of C LID+1.0: ~140* LID+0.5: ~105 LID: ~95 LID': ~50*	C: ~ 1.54 DG2: ~ 1.84 DG3: ~ 1.69 DG4: ~ 1.54 DG5: ~ 1.57	Males S: 1.06±0.14 NS: 1.10±0.18 Females S: 1.10±0.26 NS: 1.27±0.30

Furthermore, no significant changes were detected in offspring plasma for analyte T3. Analysis of samples retrieved on PND30 demonstrated significant increased concentrations of T4, and rT3 in both sexes whereas T2 was only increased in females. Analyte T4 was additionally significantly increased on PND21. Whole brain analysis of the same individual offsprings yielded a similar pattern as in plasma namely significant increased T4 and rT3 concentrations on PND30. Unlike in plasma, T3 was significantly higher in animals from the NS diet compared to the supplemented group and no significant changes were detected for T2. The following Table 36 and Table 37 summarize animal study information and TH concentrations after exposure to ID diet from exemplary literature. Table 36 focusses on literature examining the offspring after parental exposure to ID diet during gestation, and lastly Table 37 contains TH data of dams after receiving an ID diet. All the cited literature has a decline of T4 concentrations in common after administering ID diet in dams and offspring plasma which was also reflected in free T4 concentrations as reported by Zhang et al.^{105, 217}. The respective Iodide intake of ID diets ranged from 0.3 to 1.2 µg/d. Unlike T4, T3

6.4 Non-Supplementation of Iodide in Rat Diet and Its Effect on TH Concentrations

concentration in plasma of dams tended to be not significantly affected as reported by Gilbert et al. but a decline compared to control animals was reported by Versloot et al. and Zhang et al. (free T3)^{103, 217, 218}. Table 36 further includes the results in plasma of offsprings detected in this work. Concentration of Iodide intake per day of the NS diet (3.2 µg/d) was comparable to dosage group 2 and 3 (4.5 and 3 µg/d) by Gilbert et al. as well as to the mild Iodide deficient (MID) group by Zhang et al. (3 µg/d). T4 concentrations from these dosage groups reported in the mentioned literature were similar to the respective control groups on all days investigated (PND14, 21 and 40)^{103, 219}. In comparison to this work, on PND21 and PND30 a significant increase in T4 concentrations were observed, but not on PND4. T3 concentrations in offspring were reported by Gilbert et. al which were in all dosage groups similar to the control group but tended to be non-significantly increased on PND21¹⁰³. Unaltered T3 concentrations were also detected in this work. The measurement of TH concentrations in rat brain after ID diet had been rarely reported except for e.g. publications by Martinez-Galan et al. and Gilbert et al. , who both measured THs in fetal brain on GD20 or E21 and by Pedraza et al. who however did not investigate offspring brain but adult brain^{104, 135, 220}. All of these authors independent of the investigated rat age detected a decline of TH concentrations in rat brain after a low Iodide diet.

Table 36. Exemplary literature assessing TH alterations in rat offspring after parental animal exposure to ID.

“nd” was written in a cell when “no data” was available. The symbol “*” refers to reported statistical significance compared to the respective control. For calculation of Iodide intake per day of the rat, a food consumption of 20 g/d was assumed if Iodide intake was not reported as [µg Iodide/d].

Parameter/ Literature	Zhang et al. 2012 ¹⁰⁵	Gilbert et al. 2013 ¹⁰³	Zhang et al. 2016 ²¹⁹	McIntosh et al. 1981 ²²¹	This Work
Rat Species	Wistar	Long Evans	Wistar	Black hooded Wistar	Han Wistar
Iodide Intake [µg/d]	C: 5-6 LI: 1.2 3HI: 15-16	D1 (C): 20 D2: 4.5 D3: 3 D4: 1 D5: 0.5	C: 5-6 MID: 3 LID: 1.2	C: 20 ID: 0.3-0.4	S: 20 NS: 3.2
Age at Analysis	PND7 PND45	GD20 Fetus PND14 PND21	PND40	PND21	PND4 PND21 PND30
Method	Chemiluminescent immunoassay	RIA	Chemilumines- cent immunoassay	RIA	Online-SPE- LC-MS/MS
Matrix	Serum	Serum	Serum	Plasma	Plasma
T4 [nM]	PND7 C: 31.41 ±1.29 LI: 19.05 ±12.74* 3HI: 31.54 ±2.32 PND45	GD20 Fetus C: ~7.72 DG4: ~6.44* DG5: ~ 4.51* PND14 C: ~ 56.6 DG2: ~ 51.5 DG3: ~ 54.1	C: 58.95 ± 2.06 MID: 56.51 ± 2.06 LID: 30.63 ± 9.78*	C: 112.4 ±5.9 ID: 35.2 ±3.4*	Males PND4 18.35 17.13±4.50 PND21 47.12±9.32 58.98±9.34* PND30 25.83±8.24

6.4 Non-Supplementation of Iodide in Rat Diet and Its Effect on TH Concentrations

Parameter/ Literature	Zhang et al. 2012 ¹⁰⁵	Gilbert et al. 2013 ¹⁰³	Zhang et al. 2016 ²¹⁹	McIntosh et al. 1981 ²²¹	This Work
	C: 62.56 ±7.85 LI: 36.56 ±4.12* 3HI: 64.74 ±6.82	DG4: ~ 45.1 DG5: ~ 32.2* PND21 C: ~ 51.5 DG2: ~ 45.1 DG3: ~ 52.8 DG4: ~ 38.6* DG5: ~19.3*			41.64±7.67* Females PND4 18.71±2.02 17.60±3.09 PND21 48.73±9.52 60.82±8.60* PND30 24.34±6.71 37.46±3.85*
T3 [nM]	nd	PND14 C: ~ 1.23 DG2: ~ 1.31 DG3: ~ 1.21 DG4: ~ 1.23 DG5: ~ 1.15 PND21 C: ~ 1.38 DG2: ~ 1.61 DG3: ~ 1.69 DG4: ~ 1.54 DG5: ~1.61	nd	nd	Males PND4 0.45 0.43±0.083 PND21 2.19±0.18 2.31±0.19 PND30 1.90±0.29 2.12±0.16 Females PND4 0.45±0.052 0.51±0.052 PND21 2.24±0.17 2.14±0.21 PND30 0.19±0.24 1.92±0.090

Table 37. Exemplary literature assessing TH alterations in rat dams at different sampling days after exposure to ID.

“nd” was written in a cell when “no data” was available. The symbol “*” refers to reported statistical significance compared to the respective control. For calculation of Iodide intake per day of the rat, a food consumption of 20 g/d and a drinking volume of 25 mL/d was assumed if Iodide intake was not reported as [µg Iodide/d].

Parameter/ Literature	Gilbert et al. 2013 ¹⁰³	Zhang et al. 2016 ²¹⁹	McIntosh et al. 1981 ²²¹	Versloot et al. 1997 ²¹⁸	Zhang et al. 2021 ²¹⁷
Rat Species	Long Evans	Wistar	Black hooded Wistar	Wistar	Wistar
Iodide Intake [µg/d]	D1 (C): 20 D2: 4.5 D3: 3 D4: 1 D5: 0.5	C: 5-6 MID: 3 LID: 1.2	C: 20 ID: 0.3-0.4	C: 1.3 MID: 0.066 Each group pregnant & non-pregnant	KIO₃ in Drinking Water C: 2.5 LI: 0 LII: 0.125 HI: 75 HII: 250 Iodide Feed C & HI-II 7.4 LI-LII 0.29
Age at Analysis	GD16 GD20 PND21	Pre-Pregnancy GD17 PND7	PND21	GD19	PN0

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Parameter/ Literature	Gilbert et al. 2013 ¹⁰³	Zhang et al. 2016 ²¹⁹	McIntosh et al. 1981 ²²¹	Versloot et al. 1997 ²¹⁸	Zhang et al. 2021 ²¹⁷
Method	RIA	Chemiluminescent immunoassay	RIA	RIA	RIA
Matrix	Serum	Serum	Plasma	Plasma	Serum
T4 Dams [nM]	GD16 D1: ~38.62 DG4: ~32.18 DG5: ~19.31* GD20 D1: ~ 32.18 D4: ~ 25.74* D5: ~ 19.31* PND21 D1: ~ 45.05 D2: ~ 45.05 D3: ~ 47.63 D4: ~ 38.62 D5: ~ 25.74*	Pre-Pregnancy C: 44.79 ± 3.22 MID: 43.76 ± 1.67 LID: 14.16 ± 0.39* GD17 C: 31.54 ± 0.77 MID: 29.48 ± 0.90 LID: 12.87 ± 0.00* PND7 C: 37.59 ± 1.16 MID: 35.27 ± 0.77 LID: 14.29 ± 0.39*	C: 60.2 ± 6.1 ID: 8 ± 2.7*	C: 31.4 ± 0.6 C-P: 21.2 ± 1.5* MID-C: 29.5 ± 2.4 MID-P: 16.9 ± 2.0*	FT4 C: 0.004 ± 0.002 LI: 0.001 ± 0.001* LII: 0.002 ± 0.001 HI: 0.0056 ± 0.001 HII: 0.0059 ± 0.003*
T3 Dams [nM]	GD16 D1: ~1.38 D4: ~1.31 D5: ~1.38 GD20 D1: ~1.38 D4: ~1.41 D5: ~1.38 PND21 D1: ~1.15 D2: ~1.38 D3: ~1.37 D4: ~1.35 D5: ~1.45	nd	nd	C: 0.55 ± 0.04 C-P: 0.49 ± 0.03* MID-C: 0.69 ± 0.06 MID-P: 0.47 ± 0.04*	FT3 C: 0.002 ± 0.0004 LI: 0.0007 ± 0.0003* LII: 0.0011 ± 0.0004* HI: 0.0017 ± 0.006 HII: 0.0014 ± 0.0005*

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6.5.1 TH Concentrations in the Developing Zebrafish

In section 5.9.1 results from TH analysis in pooled zebrafish (*Danio rerio*) embryos were described. After first testing whether TH concentrations were measurable in pooled embryos and with what intensity, a proof-of-concept study with PTU was conducted. Results showed an overall decrease of T3 concentrations in control embryos over age as well as no significant changes due to PTU exposure. On contrary, T4 concentrations in control animals increased strongly at 96 hpf compared to the other ages. Due to the strong T4 increase on 96 hpf in control embryos and the similar T4 concentrations in PTU exposed embryos over all timepoints,

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significant lowered T4 concentrations were thus estimated at 96 hpf. Thyroid synthesis of T4 has been detected at around 70 to 80 hpf which might explain the detected T4 increase in the presented results¹⁴¹.

The tested PTU concentrations in this study were adapted from a previous conducted zebrafish study focusing on the morphological and metabolic changes after PTU exposure but these concentrations were also within a similar range of other reported PTU studies in zebrafish which were e.g. set as follows: 0, 0.29, 0.59, or 1.47 mM^{120, 222}. Metabolic changes were visible in 'phenylalanine, tyrosine and tryptophan biosynthesis' pathway, and changes of tyrosine and pipecolic acid were linked to the MOA of PTU as it inhibits TPO and thus TH synthesis²²². The study itself did not include TH measurements in the pooled embryos, but TH alterations were assumed from the detected metabolic changes and from literature research. The second mentioned paper referring to similar tested PTU concentrations focused on the assessment of retinal development, visual function, and subsequent swimming behavior whereas TH concentration was not determined either¹²⁰. This assumption was e.g. thoroughly reviewed in the corresponding adverse outcome pathway (AOP) report for TPO inhibition (AOP 363) which linked the decrement of T4 and T3 in serum to altered retinal layer structure to altered visual function ultimately causing increased mortality and thus decreased population growth rate²²³. Therefore, the authors suggested that morphological and structural examination during fish eye development shall be included as an endpoint in ED test guidelines to determine substances as THD²²³. Impaired visual function by different THD had been previously shown in zebrafish embryos. Direct TPO inhibitor PTU and thyroid action disruptor TBBPA exposure led to decreased total body length and eye size at 120 hpf consequently leading to visual impairment identified by swimming behavior and optokinetic response¹²⁰. Changes in gene expression levels further revealed that PTU significantly caused an upregulation of TPO but TBBPA a downregulation¹²⁰. However, both substances non-significantly tended to upregulate TSH transcripts¹²⁰. Decreased TH concentrations in adult zebrafish with increasing PTU concentration have been reported^{224, 225}. With regards to a better comparison with the here established concentrations the focus lied on literature reporting TH concentrations from zebrafish embryos (≤ 120 hpf). As for example TH concentrations in zebrafish embryo at 120 hpf have been reported to be 5.98 ± 0.06 pg/larvae for T4, 0.44 ± 0.17 pg/larvae for T3, and 0.16 ± 0.06 pg/larvae for rT3 in a publication by Chen et al.²²⁶. On contrary, on 24 hpf T4 concentration were detected to be around 0.25 pg/embryo and around 0.3 pg/embryo T3¹²³. Walter et al. detected T4, T3, rT3, and T2 in 150 pooled zebrafish embryos per sample collected at 24, 72, and 120 hpf using a LC-MS/MS method whereas T2 concentration in all samples was

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detected to be below the LOD of 0.003 pg/embryo²²⁷. Exposure was conducted with 30 embryos per well to a final concentration of 0.01 % PTU (725 μ M originated from 1 % stock solution that yielded 72.5 mM) which were afterwards pooled to 150 embryo per sample. Walter et al. detected a decline of T3 concentration from 24 hpf to 120 hpf whereas T4 concentration increased. The corresponding calculated T3 concentrations decreased from 0.37 ± 0.05 pg/embryo at 24 hpf to 0.18 ± 0.02 pg/embryo at 72 hpf to 0.12 ± 0.007 pg/embryo at 120 hpf²²⁷. This decline of T3 concentrations was consistent with the presented T3 results yielding 0.398 ± 0.152 pg/embryo at 24 hpf to 0.309 ± 0.133 pg/embryo at 48 hpf to 0.223 ± 0.050 pg/embryo at 72 hpf to 0.111 ± 0.035 pg/embryo at 96 hpf in this work. T4 concentration was reported to be 0.1 ± 0.01 pg/embryo at 24 hpf which increased approximately 10-fold by 72 hpf and a further 50-fold by 120 hpf²²⁷. Similar to the rise in T4 concentrations with age, rT3 concentrations were detected to increase simultaneously from 0.09 ± 0.05 pg/embryos at 24 hpf to 0.3 ± 0.01 pg/embryos at 120 hpf which was concluded to indicate that more T4 was deiodinated to rT3²²⁷. Corresponding exposure to 0.01 % PTU led to lower concentrations of T3 but not significantly at 72 hpf and 120 hpf, T4 concentrations significantly declined at 72 hpf and at 120 hpf as well as rT3 significantly decreased at 120 hpf²²⁷. A tendency of the increase of T4 with time was also detected in the depicted results, however to a lesser extent of around 3-fold from 24 hpf to 96 hpf. The analysis in this work did not identify rT3 which might be due to the lesser number of pooled zebrafish embryos compared to the cited literature (50 vs 150). Moreover, exposure to various concentrations of PTU did not cause significant decreased T3 concentration compared to the respective control but a decline of T4 was significant at 96 hpf for all tested PTU concentrations similar to the pattern cited for Walter et al. Further, Walter et al. tested a single concentration of 0.01 % PTU which was generated from a 1 % stock solution set at 72.5 mM thus yielding a tested concentration of 725 μ M (0.01 %) causing a decline in T4 concentration as well as tendency of T3 reductions²²⁷. This reduction was reproduced for T4 at already the lowest tested concentration in this work at 325 μ M indicating the strong TPO inhibitory potency of PTU. Therefore, it should be considered to repeat the study including a lower concentration range to detect whether a dose-response curve for PTU in pooled zebrafish embryos can be derived. With regards of repeating the study, it is additionally important to reconsider the temperature of the water which has an influence upon zebrafish development as thoroughly reviewed by López-Olmeda and Sánchez-Vázquez²²⁸. The most used water temperature is 28.5 °C which was also applied in the cited literature above, however in this study the temperature was set at 26 °C. Even though zebrafish embryo develops normally within a temperature range of 25-33 °C²²⁹, it is a distinguished

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parameter setting which might have influenced onset of T4 synthesis explaining the delayed increase in T4 concentration starting at 96 hpf compared to the above cited literature of Walter et al. at 72 hpf.

One further difference between the here presented sample preparation and the one by Walter et al. is the addition of 20 mg pronase after homogenization of the fish embryos which they adapted from the procedure of Chen et al.²²⁶. Publication of Chen et al. entailed detailed sample preparation description involving 16 h incubation with pronase buffer, extraction using cold acetone, sample purification utilizing offline SampliQ SPE cartridges, derivatization of the dried residue followed by evaporation and final resuspension in 100 μ L MeOH which was filtered and 5 μ L injected into a LC-MS/MS system²²⁶. Despite bigger differences in sample preparation, results were good comparable but could be a reason for the difference in number of analytes detected. However, it should be considered first to increase the number of pooled zebrafish embryos as well to e.g. 75, 100, or 150 since on the one hand peak intensity for all analytes would improve and on the other hand additional analytes such as rT3 and T2 could be captured.

To summarize, the developed method is suitable for the detection of alterations of TH concentration in pooled zebrafish embryos that were exposed to THD yielding comparable results to literature. However, improvements with regards to increasing the number of pooled zebrafish embryos to e.g. 75, 100 or even 150 should be considered. This will increase peak intensity as well as it will enable the possibility to detect further analytes such as rT3 and T2 which will obtain further information about e.g. DIO conversion patterns within the organism and possible inhibition by tested substances. Another important parameter to adjust is the water temperature which should be set at 28.5 °C to optimize zebrafish development. A repetition of the study with PTU at a water temperature of 28.5 °C will most importantly verify whether an increase of T4 at already 72 hpf similar to reported values from Walter et al. is obtainable which would emphasize the improved development of the zebrafish embryos. This repetition should be combined with testing lower PTU concentrations below 325 μ M to assess whether a dose-dependent T4 decline by PTU is derivable whereas e.g. the two highest concentrations from this experiment could be omitted. After improvement and evaluation of the mentioned parameters the testing scheme shall be expanded to substances with different TH-related MOAs and potencies so that their TH alteration pattern can be estimated which might be helpful to assess TH-related MOAs for new substances. Some examples might be ETU, MMI, or substances interacting by indirect TH-related MOAs such as liver enzyme inducers or TBBPA. Therefore, species differences should be kept in mind since e.g. PTU does not inhibit DIO1

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such as in mammals²³⁰. The cited AOP 363 as mentioned above links TPO inhibition to decreased T4 and T3 in serum for which evidence was provided in this work and in the cited literature for T4. However, no significant decline was estimated for T3 even though the tendency of lowered concentration is present. TH concentrations in zebrafish embryos are usually detected as whole-embryo concentration because of the infeasibility of obtaining serum and thus portrays a proximity to the concentration found in serum. However, when combining analysis of TH concentration in zebrafish embryos with further endpoints such as eye development, specific gene expression, or changes in behavior as e.g. in swimming, it provides a favorable model for the assessment of THD as already suggested in literature¹²⁶.

6.5.2 Determination of rT3 and T2 Concentrations to Estimate DIO1 Inhibition in Human Liver Microsomes

As mentioned in section 5.9.2.1 the inhibitory potential of a substance on DIO1 is usually photometrically evaluated utilizing the SK reaction. Limitations of this assay are however other reducing substances as for example ferrous ion or thiocyanate¹⁴² but also Iodide-containing substances. That is why a confirmatory analysis of these substances was performed using the described online-SPE-LC-MS/MS method to directly measure substrate rT3 and product T2. In both methods no IC₅₀ was derived for the following substances emphasizing their lack of DIO1 inhibitory potential: Ketoconazole, Divanadium Pentoxide, Resorcinol, and TMTU as it was previously detected by Weber et al.¹³⁰. The substances Amiodarone and TETRAC structurally contain Iodide and thus they could not be evaluated using DIO1-SK assay but with direct online-SPE-LC-MS/MS analysis. The subsequent IC₅₀ values were 8.05 μ M and of 3.75 μ M for T2 respectively. This reflected an advantage of online-SPE-LC-MS/MS analysis since its independent from any interference by the test substance or by buffer ingredients disturbing DIO1 reaction in an unwanted or untargeted manner. IC₅₀ results for the remaining substances obtained from both methods are listed in Table 38 below.

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Table 38. Range of IC₅₀ values of the tested substances estimated from both methods for each biological replicate individually.

The concentration range involves results from two biological experiments for which each concentration was examined in three technical replicates. The concentration range includes the IC₅₀ values which were calculated individually for each biological replicate. No curve could be fitted from the TBBPA data obtained for rT3 and the Silichristin data for T2.

Substance	Min-Max IC ₅₀ [μM]		
	DIO1-SK	rT3	T2
PTU	11.7-15.5	1.8-4.2	2-3.6
BPA	13.5-24.4	15.8-22.7	13.2-22.5
Desipramine	2.2-6.8	1-1.2	1.2-5.2
Linoleic Acid	13.2-34.9	27.1-113.2	64.7-71.1
Silichristin	143.5-153.5	125-156.8	/
Tannic Acid	10.8-11.5	5.4-9.3	7.8-8.6
TBBPA	9.5-10.8	/	7.8-9.4

From the data of Niflumic acid and Rosmarinic acid no inhibitory concentrations were derived due to insufficient fitting of the concentration curve. In future experiments this could be prevented by adding one or two more test concentrations. This suggestion was underlined by the comparison of curve fitting from experiments with Amiodarone in which either four or eight concentrations were tested as depicted below in Figure 69. As it was visible by comparing both graphs, the adjustment to eight tested concentrations improved the fitted curve for in particular T2 which subsequently enabled to calculate the IC₅₀ value. The adaption of tested concentrations could be further optimized for Rosmarinic acid, and Niflumic acid to better capture the plateaus of the curves. As well as for Silichristin more concentration steps above 100 μM would have been necessary to potentially better estimate a value for T2.

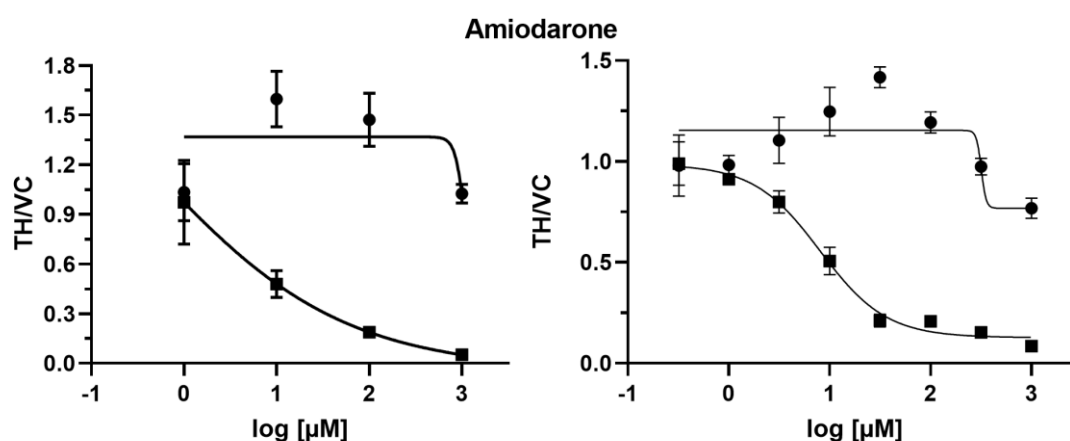


Figure 69. Comparison of curve fitting between four and eight tested concentrations of Amiodarone.

Both graphs plot the ratio of TH and the vehicle control on the y-axis and the tested substance concentrations on the x-axis. Data of analyte rT3 are symbolized with a dot whereas T2 data with a square. Left graph demonstrates the results with four tested concentrations which were further derived from two biological replicates. Data from the right graph involves one biological replicate and eight tested concentrations of Amiodarone.

6.6 Final Evaluation of Developed Online-SPE-LC-MS/MS Method for TH Analysis

Besides that, both methods reached similar results for the listed substances from which it was possible to derive an IC_{50} value. Slightly bigger differences between the methods were found for PTU and Linoleic acid. Online-SPE-LC-MS/MS analysis for PTU reached an overall lower range compared to the DIO1-SK assay whereas an increased range for Linoleic acid over DIO1-SK results were detected. However, the two independent experiments for Linoleic acid resulted in a large deviation of rT3 data whereas for T2 the outcome from both experiments were generally closer emphasizing the assumption of T2 being a better readout parameter over rT3. PTU was chosen as a reference substance since it is known *in-vivo* to inhibit DIO1. For around half of the tested substances, an inhibitory potential using either method was derived which was indicated by the obtained IC_{50} values listed in Table 38¹³¹. The respective substances reflected different inhibitory potentials from strong to mild effects when comparing T2 derived IC_{50} values to the estimated value of PTU. A similar strong effect to PTU was detected for Desipramine. Moreover, Tannic acid and TBBPA yielded similar results, BPA was slightly higher whereas Linoleic acid had the highest IC_{50} . No T2 based IC_{50} was derived for Silichristin whereas the respective rT3 IC_{50} values were comparable between the methods.

Ultimately, direct analysis of substrate rT3 and product T2 via online-SPE-LC-MS/MS enabled a robust and reliable method to identify DIO1 inhibitory potential of substances tested in this study in human liver microsomes. Online-SPE-LC-MS/MS analysis is independent from any interferences such as e.g. by Iodide-containing substances that would cause false positive results in the DIO1-SK assay due to the release and consequently detection of the Iodide from the substance which cannot be differentiated from Iodide released by rT3 conversion. Besides these conflicts both methods yielded all in all comparable IC_{50} values for the remaining tested substances. Within the framework of the joint research program by EURL ECVAM, DIO1 inhibition was planned to be evaluated via the SK reaction and thus this study was conducted to compare and confirm the DIO1-SK assay results with online-SPE-LC-MS/MS analysis.

6.6 Final Evaluation of Developed Online-SPE-LC-MS/MS Method for TH Analysis

In the course of this work various methods to detect THs were sufficiently developed obtaining robust and reliable results. The primary focus was on TH analysis in rat plasma and brain for which methods were additionally validated. An advantage of LC-MS/MS is the simultaneous analysis of multiple analytes, therefore each developed method in this work included at least analytes T4, T3, rT3, T2, T1 as well as their isotope-labelled analogues used for quantification. This enables an unified overview of each affected TH analyte but also allows for insight into general TH conversion and possible disruption of the responsible conversion

6.6 Final Evaluation of Developed Online-SPE-LC-MS/MS Method for TH Analysis

enzymes (DIO1, DIO2, and DIO3) in tissue. For completion of TH-related important parameters in rat plasma, an experimental trial was conducted with the aim to analyze TSH using LC-quadrupole time-of-flight MS (LC-Q-TOF MS). The introduction of the PureProteome™ Albumin/IgG Depletion Kit in sample preparation procedure did reduce albumin concentration in the sample verified by the consequent detection of low abundance proteins that were not detected prior. However, current state of the analytical method was not sufficient to identify TSH in rat plasma due to its low abundance, thus further method development is needed. Further improvements might be achieved using a nano-LC which due to the low flow rate of the mobile phases might increase separation of the proteins and thus increase sensitivity. To facilitate detection of the corresponding TSH peak, chromatographic separation and thus TSH retention time should be identified using recombinant TSH protein. This is due to the possibility of adjusting the concentration needed for detection since TSH concentration in plasma is low (~1.5-2 ng/mL) as derived from literature^{103, 135}.

Firstly, TH plasma method development was completed and subsequently plasma samples from rats exposed to substances acting through various TH-related MOAs analyzed which revealed good comparability with reported literature as well as differences between the MOAs could be identified. This as well as the consistent sample preparation indicated by the technical replicates of the plasma pools included in every analysis further underlined robustness and reliability of the analytical method. The addition of more analytes besides T4 and T3 revealed the tendency that reductions in T4 are mirrored to a similar extent in rT3 concentrations in rat plasma. As seen in Table 23 T4 and rT3 were similar affected by Aroclor 1254 exposure by 90 %, by TBBPA by around 70 %, by PFHxS by around 86 % whereas T3 was reduced by 40 %, 45 % or 60 % respectively compared to the given control. This similarity between T4 and rT3 was negligible for strong inhibitors such as PTU and MMI. Furthermore, the typical difference of higher T4 concentration in male adult rats than in female adult rats was also visible in rT3 concentration but neither for T3 nor T2 which is best seen in Figure 20.

Secondly, the TH plasma method was adjusted for analysis of rat brain which at the beginning consisted mainly of generating control TH data in various rat ages since literature is rare regarding rat whole brain TH analysis. Interestingly, analyte as well as age-specific differences in whole brain were observed such as peak concentrations of T3 and T2 were reached on PND21, but for rT3 already on PND4. This was reproducible for T3 and more clearly for T4 in rat plasma on PND21 whereas rT3 and T2 were unchanged in plasma at every age. The concentrations of rT3 and T2 were overall higher and thus more easily detectable in rat brain than in plasma. This difference between brain and plasma reveals the relevance of including

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more TH analytes in tissue analysis which due to local DIO conversion leads to a more nuanced distribution of TH concentrations. When analyzing specific brain regions (cerebellum and cortex), analyte as well as age dependency was observed again. This was marked by different T3 and T2 concentrations in each region on PND4, but similar on PND21 and forward. On contrary rT3 concentrations were similar on PND4, different on PND21 and in adult rats similar again. Only detected concentrations of analyte T4 did not differentiate. These described patterns were reproducible between several studies.

The benefit of analyzing more TH analytes than T4 and T3 was further emphasized by the detection of DIO1 inhibition *in-vitro* (results section 5.9.2). In this approach DIO1 activity after incubation to inhibitors in human liver microsomes was assessed by adding rT3 as the substrate due to its higher affinity to the enzyme compared to the remaining THs. To apply this approach no additional method development was needed except for adjusting the calibration range to the expected concentrations because the online-SPE-LC-MS/MS method already included the required analytes. This highlights the flexible adaption to TH analysis in various matrices due to having an analytical method that includes the main TH metabolites to e.g. cover TH conversion. Simultaneously to fast analysis of rT3 to T2 conversion, a possible T4, T3, or T1 background in human liver microsomes was denied since these analytes were not detected. Therefore, the downstream metabolites of T4 and T3 in the online-SPE-LC-MS/MS method are crucial parameters for assessing DIO conversion in either *in-vitro* approaches or even potentially *in-vivo* in different tissues.

Overall, the inclusion of additional TH analytes besides T4 and T3 in the developed online-SPE-LC-MS/MS method enabled a broad insight of each TH concentration in various matrices and rat ages. Even though, in rat plasma rT3, T2, and T1 might not be the most relevant parameters since T4 and T3 are the major regulators of the negative feedback mechanism and biological activity, they did however align with the reductions detected in rat plasma. Especially, rT3 seemed to be equally affected as T4 in rat plasma. The major advantage however is the assessment of TH conversions in matrices either *in-vitro* or *in-vivo* for which the downstream TH analytes are additional indicators. This was underlined by the TH analysis conducted in rat brain and human liver microsomes in this work.

7 Conclusion & Outlook

Taking everything together, robust and precise online-SPE-LC MS/MS methods have been developed to detect THs in rat brain and plasma which was underlined by the shown validation results. Additionally, the plasma method was further applied by detecting TH concentrations in plasma samples received from animals exposed to various substances with TH-related MOAs and it showed similarity to cited literature. The included TH-related MOAs were TPO inhibition, NIS inhibition, liver-enzyme induction as well as the competitively binding to plasma TH-binding proteins. The strongest reductions in plasma TH concentrations were detected for TPO inhibitors followed by the substances interfering with plasma TH-binding proteins. Plasma TH analysis was expanded towards analysis of the free fraction using RED. The addition of IS during RED led to an overall 10 times higher concentrations of fTHs compared to cited literature or compared to afterwards addition of the IS-Mix which is caused by the subsequent concentration calculation including the area ratio of the given analyte and the IS which was consequently higher when the IS was added during RED due to the area loss during the procedure. Substance induced decline of fTHs by PTU or Aroclor 1254 was within a similar potency as reported in the literature. Regarding fTH analysis further substances with different TH-related MOAs should be tested as already done for total TH concentrations. The focus should be on substances particularly interacting with the plasma TH-binding proteins (e.g. TBBPA) to verify whether differences between free and total fractions are detectable or whether alterations are simultaneous. This would further clarify whether analysis of fTH concentrations in rat plasma is a necessary diagnostic parameter. So far - due to the high volume needed (200 μ L) - it is more likely that fTHs will not be assessed routinely. As mentioned in section 6.6 the attempt to analyze TSH in rat plasma by LC-quadrupole time-of-flight MS (LC-Q-TOF MS) was not sufficient to securely identify TSH and could be pursued further with the mentioned suggestions.

TH analysis in rat brain primarily consisted of control animals to establish physiological TH concentrations in whole-brain, cerebellum and cortex on PND4, PND21, and in adult rats which was analyzed in parallel in plasma of the same individual animals. With increasing age of the rat, it was detected that T3 yielded peak concentrations in both matrices on PND21 whereas T4 tended towards the same pattern, but it was less notable in brain than plasma, however rT3 and T2 were unchanged in plasma but did show age dependency in brain. This is an important observation regarding THs dynamic adaption of concentration during (neuro-)development and the return to a lower concentration in the adult rat. TH analysis in brain was expanded to TH

analysis in specific brain regions, but also to the first testing of TH analysis from paraffin-embedded and formalin-fixed half-brain. Including more brain regions for analysis will reveal in depth knowledge of TH dynamic during development, however due to similar concentrations of T4, T3 and T2 in the analyzed regions on PND21 it is questionable whether brain-region specific analysis would be preferred over whole brain analysis potentially needed in regards to e.g. regulatory assessment. On the contrary, analysis from fixed tissue would create more possibilities for TH assessment in retrospect which first testing however did not yield the expected concentrations in rat brain and needs further investigation. Further method development could include a better removal of paraffin or testing of the possibility to adapt the MS method to the TH bound to Formaldehyde masses to circumvent insufficient rehydration of the respective tissue.

Furthermore, Iodide supplemented, and non-supplemented diet given to parental animals four weeks prior mating until PND21 did not cause significant reductions in plasma nor brain TH concentrations in the offspring. However, a significant incline in plasma T4 and rT3 as well as brain T4, T3, and rT3 concentrations were manifested on PND30. Since the non-supplemented diet still was within the dietary requirement of 150 to 200 µg/kg and thus cannot be defined as Iodide deficient (~ 20 µg/kg) a subsequent animal study should be conducted. In this study diets should be below the Iodide dietary requirement to examine the consequences of Iodine deficiency during development of the offspring. An overview of already conducted Iodine deficiency studies including the calculated Iodide intake was tabularized in section 6.4 which could guide further study planning.

During the scope of the project, this method was easily adjusted to further matrices not concerning the rat such as human liver microsomes and zebrafish embryos. This underlines the flexibility of TH analysis for which the online-SPE-LC MS/MS method functions as a basis element and just prior sample preparation needs to be adjusted for the given matrix. This is underlined by the broad spectrum of TH analytes included in the method ranging from the educts MIT and DIT to the excretion metabolites T4-Gluc and T4-SO₄. Thus, another suggestion is to investigate further tissues such as e.g. thyroid gland and/or the liver to widen the TH-related analysis and covering more tissues that could be affected by various TH-related MOAs. Such as e.g. DIO inhibition was assessed in human liver microsomes within this work by comparing analysis of DIO1 conversion detected with SK reaction or online-SPE-LC-MS/MS which revealed in general good comparability between the methods. An advantage of direct analysis of rT3 and T2 by online-SPE-LC-MS/MS is that the method is less susceptible to interferences by e.g. buffer ingredients or Iodide-containing substances making this analysis

more reliable, but its usage is restricted for a wide range of laboratories due to the instrumental requirements.

Lastly, first analysis of TH whole embryo content of the zebrafish (*Danio rerio*) obtained promising results in the control group but also in the groups exposed to various concentrations of PTU which demonstrated equivalent results to other reports. However, there are still some parameters that should be optimized in the future such as the testing of a higher number of pooled zebrafish embryos (e.g. 75 to 150) leading to the detection of more TH analytes, repetition of experiment with water temperature to 28.5 °C to compare TH concentrations as well as the development of zebrafish embryos, and finally the testing of more THDs to verify whether less strong effects caused by TH-related MOAs are also detectable.

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Curriculum Vitae

Education & Experience

SEP 2021- JULY 2025	PhD Project BASF Metabolome Solutions GmbH & University of Kaiserslautern-Landau (RPTU Kaiserslautern-Landau), Germany • Development of Analytical Methods to Quantify Thyroid Hormones (TH) in Multiple Biological Matrices to Facilitate the Prediction of Endocrine Disruption in the Testing and Assessment of Chemicals Continuing Education in Toxicology • Analytical Toxicology • Clinical Toxicology • Immunotoxicology • Reproductive and Developmental Toxicology by European Teratology Society • Molecular cell toxicology • Toxicokinetic • Basics of Ecotoxicology
OCT 2018 – MAR 2021	Master of Science “Toxicology” Heinrich-Heine-University, Düsseldorf, Germany • Lab animal training (FELASA certificate F048/16) • Accomplished equivalent courses for the registration as toxicologist: • Organ Toxicology I & II • Regulatory Toxicology
MAR 2020 – DEC 2020	Master Thesis Project University of Florida, Gainesville, USA • Sulfonation studies of hydroxylated bromodiphenyl ethers in two fish species • Master Thesis: LC-MS/MS method development to determine if bromodiphenyl ethers inhibit thyroid hormone formation in animal livers
MAR 2018 – Sep 2018	Internship Bayer AG (Research & Development), Cologne & Wuppertal, Germany • Measurement of physical-chemical properties of a certain set of drugs & testing of this set using the AC-SINS assay
Sep 2017 – FEB 2018	Internship - Continued work based on topics of Bachelor Thesis Centrum for Molecular Medicine (CMMC), Cologne, Germany
SEP 2014 – FEB 2018	Bachelor of Science “Pharmaceutical Chemistry“ University of Applied Science, Cologne, Germany • Bachelor Thesis: Mutation of antibody binding domains of chimeric antigen receptors (CAR) focusing on T-cell-activation in cellular immunotherapy
2024	Conferences • Poster Presentation at EUROTOX Congress, Copenhagen, Denmark

- 2023
- Speaker at Satellite Symposium “Testing, Assessment and Classification of Thyroid Hormone Disruptors” ahead of the EUROTOX Congress
 - Poster Presentation at EUROTOX Congress, Ljubljana, Slovenia
- Publications**
- 2023/2024
- Stefanidis, K., N. Hambruch, K. Renko, **C. Hindrichs**, T. Walk, H. Kamp, D. Funk-Weyer and R. Landsiedel (2024). Assessment of the Robustness and Accuracy of the Deiodinase 1 Sandell–Kolthoff Assay: A Comparative Analysis. Applied In Vitro Toxicology **10**(4): 80-90.
- Hindrichs, C.**, Walk, T., Landsiedel, R., Kamp, H., Schneider, S., Melching-Kollmuss, S., & Funk-Weyer, D. (2024). Thyroid Hormone Metabolites Quantified in Pup and Adult Rat Cerebellum, Cortex and Whole- Brain Samples Using an Automated Online SPE-LC-MS/MS Method. *Metabolites*, 14(1), 61. <https://doi.org/10.3390/metabo14010061>
- Hindrichs, C.**, Walk, T., Melching-Kollmuss, S., Landsiedel, R., Kamp, H., & Funk-Weyer, D. (2024). A Novel and Fast Online-SPE-LC-MS/MS Method to Quantify Thyroid Hormone Metabolites in Rat Plasma. *Chemical research in toxicology*, 37(1), 33–41. <https://doi.org/10.1021/acs.chemrestox.3c00152>
- 2022
- Sultan, A., **C. Hindrichs**, K. V. Cisneros, C. J. Weaver, L. R. Faux, V. Agarwal and M. O. James (2022). Hepatic demethylation of methoxy-bromodiphenyl ethers and conjugation of the resulting hydroxy-bromodiphenyl ethers in a marine fish, the red snapper, *Lutjanus campechanus*, and a freshwater fish, the channel catfish, *Ictalurus punctatus*. *Chemosphere* 286(Pt 1): 131620.