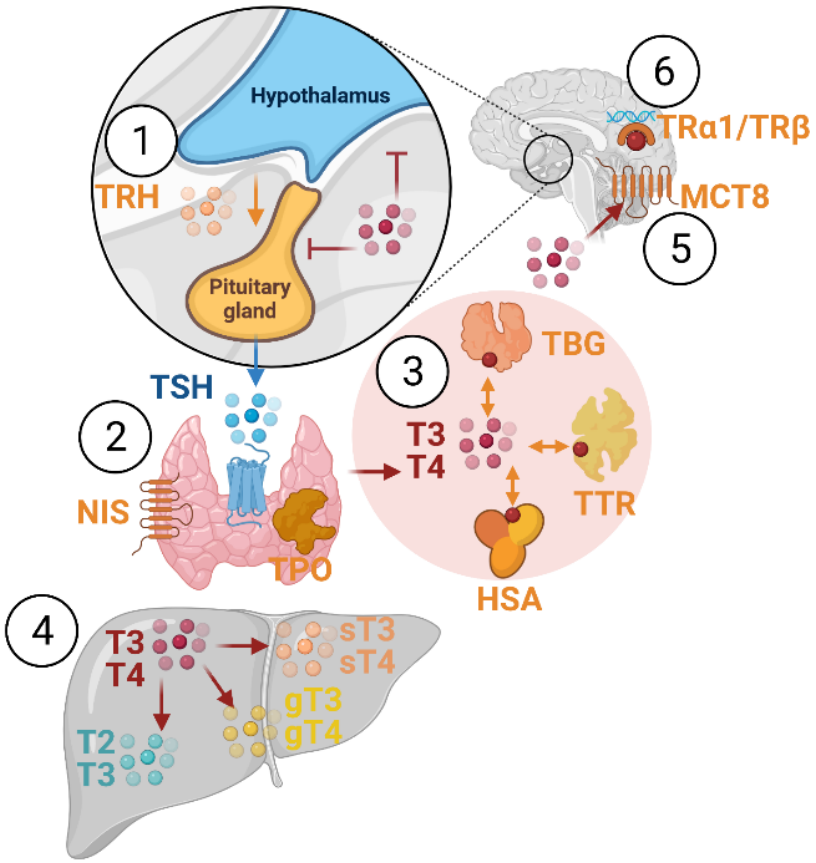
STUDY REPORT

for the TPO-catalyzed tyrosine iodination assay using liquid chromatography – Part 1

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



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This study report has been prepared within the context of a collaboration agreement signed in 2019 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. For information on the methodology and quality underlying the data presented in this report, users should contact the referenced source.

This study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed and experimentally assessed by EU-NETVAL laboratory Charles River Laboratories, Den Bosch.

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FINAL REPORT

Test Facility Study No. 20309164

In Vitro Suppression of Thyroid Peroxidase (TPO)-Catalyzed Iodination using FTC-238-hrTPO Cell Homogenates

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QUALITY ASSURANCE STATEMENT

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No. 20309164

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study				
	Final Study Plan	31-Aug-2021	31-Aug-2021	31-Aug-2021
	Addition of Test Material to Invitro Test System	28-Sep-2021	28-Sep-2021	28-Sep-2021
	Report	30-Dec-2021	04-Jan-2022	04-Jan-2022
	Final Report	07-Jun-2022	07-Jun-2022	07-Jun-2022
Process				
	In Vitro ADME	06-Jul-2021	15-Jul-2021	15-Jul-2021
		19-Oct-2021	28-Oct-2021	28-Oct-2021
	Test Item Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Test Item Receipt	21-Jun-2021	21-Jun-2021	21-Jun-2021
		07-Sep-2021	21-Sep-2021	21-Sep-2021
	Test Item Handling			

*TFM=Test Facility Management SD = Study Director

All electronic signatures appear at the end of the document upon finalization.

COMPLIANCE STATEMENT AND REPORT APPROVAL

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI) and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions from the above regulations are listed below.

- Concentration, stability, and homogeneity of test item formulations were not determined in this study. However, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use and all preparations were used within 4 hours after preparation of the formulation.

This study was conducted in accordance with the procedures described herein. All deviations authorized/acknowledged by the Study Director are documented in the Study Records. The report represents an accurate and complete record of the results obtained.

There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

All electronic signatures appear at the end of the document upon finalization.

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Jelle Reinen, PhD, ERT	Address as cited for Test Facility
Test Facility Management	Charles River	Beppy van de Waart, MSc, ERT	Address as cited for Test Facility
Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility

2. SUMMARY

This study was performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of method 2C (Tyrosine iodination using liquid chromatography) in standard operating procedures (SOPs), in this study the robustness and reliability of the method to determine the suppression of human thyroid peroxidase (TPO)-catalyzed iodination was assessed *in vitro*. This was done by performing five valid runs with the reference item 6-propyl-2-thiouracil (PTU, CAS# 51-52-5) and the test items flavanone, methimazole, N,N,N,N-tetramethylthiourea (TMTU), naringenin and sulfamethazine.

FTC-238-hrTPO cells are human thyroid carcinoma cells stably transfected with an expression clone coding for human recombinant (hr) TPO and can be used to prepare cell lysates containing the hrTPO enzyme. To evaluate potential interference with TPO-catalyzed iodination, FTC-238-hrTPO cell lysates can be incubated with L-tyrosine, potassium iodide and hydrogen peroxide (H_2O_2) in the presence or absence of a test item. During incubation, TPO enzymatically converts L-tyrosine into monoiodotyrosine (MIT) and formation of this metabolite can be monitored by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as a direct measurement of TPO-catalyzed iodination. A small amount of MIT may also be formed non-enzymatically, its formation will be assessed in separate incubations.

Based on the solubility assessments, it was decided to test each test item at concentrations of 10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM, 31.6 µM and 100 µM during the initial TPO-catalyzed iodination experiment. For the remainder of the experiments, TMTU, flavanone and sulfamethazine were tested at the same concentrations whereas for methimazole (3.16 nM, 10 nM, 31.6 nM, 100 nM, 316 nM, 1 µM, 3.16 µM and 10 µM) and naringenin (10 nM, 31.6 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM and 31.6 µM) the concentration range was adjusted.

In total five valid and independent TPO-catalyzed iodination experiments were performed. During each experiment, the test items were tested at eight concentrations (in triplicate) together with vehicle controls, no-vehicle controls, no-peroxide controls, non-enzymatic iodination controls, the negative control inhibitor bis-(2-ethylhexyl)-phthalate (DEHP, CAS# 117-81-7) at a single concentration and a complete dose-response curve for the reference item PTU. Dimethyl sulfoxide (DMSO) was used as the vehicle and the concentration of vehicle in the incubations was kept constant at 1% (v/v).

For each of the experiments, the percent TPO-catalyzed iodination compared to the vehicle control was ≥ 92% for the negative control DEHP which was well above the acceptance criterion of > 80%. For each of the experiments, the mean MIT concentrations of the no-peroxide controls were always below the LOQ (0.0761 µM) and were therefore considered background. These results confirmed that MIT formation in the incubations was dependent on the presence of H_2O_2. For each of the experiments, the mean percent TPO-catalyzed iodination of the no-vehicle controls when compared to the average TPO-catalyzed iodination in the vehicle control samples varied between 98% and 111% and thus was within the range of 80%-120%. As all acceptance criteria for the controls were met, the TPO-catalyzed iodination experiments were considered valid.

For each of the five valid experiments, the curves for the reference item PTU were sigmoidal and were composed of a minimum of six concentrations. The reference item PTU suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 1.21 µM up to 1.94 µM which was within the acceptance range of 5×10^{-7}-5×10^{-6} M. The averaged IC_{50} value for all experiments was found to be 1.61 µM. As all acceptance criteria

for the reference item PTU were met, the TPO-catalyzed iodination experiments were considered valid.

The averaged results of the five valid experiments for the reference control PTU and the test items are summarized in the Text Table below.

Text Table 1
Curve Fit Parameters, Log IC_{50} and IC_{50} Values obtained for the Reference Control PTU and the Test Items

Parameter	PTU	TMTU	Methimazole	Naringenin	Flavanone	Sulfamethazine
Bottom (%)	-0.7556	-0.4361	1.126	-1.663	No inhibition	-9.762
Top (%)	106.6	105.1	1.305	105.1		106.7
Log IC_{50} (M)	-5.794	-5.821	-6.415	-6.355		-4.942
HillSlope	-2.412	-2.323	-3.789	-1.421		-0.9569
IC_{50} (µM)	1.606	1.509	0.3842	0.4420		11.43
Values	118	120	95	120		109

In conclusion, five valid TPO inhibition experiments were performed. The test items TMTU, methimazole, naringenin and sulfamethazine were found to be positive and suppressed the human TPO-catalyzed iodination in a dose-dependent manner. The test item flavanone was found to be negative and did not suppress the human recombinant TPO-catalyzed iodination. By performing these experiments, it was demonstrated that the developed method to determine the suppression of human recombinant TPO-catalyzed iodination *in vitro* was robust and reliable.

3. INTRODUCTION

This study was performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of method 2C (Tyrosine iodination using liquid chromatography) in standard operating procedures (SOPs), in this study the robustness and reliability of the method to determine the suppression of human thyroid peroxidase (TPO)-catalyzed iodination were assessed *in vitro*. This was done by performing five valid runs with the reference item 6-propyl-2-thiouracil (PTU, CAS# 51-52-5) and the test items flavanone, methimazole, N,N,N,N-tetramethylthiourea (TMTU), naringenin and sulfamethazine.

TPO is an enzyme which is present on the apical membrane of thyroid follicular cells where it reduces hydrogen peroxide (H_2O_2), thereby elevating the oxidation state of iodide to an iodinating species (often considered to be hypoiodous acid), and iodinates tyrosyl residues in the thyroglobulin (Tg) glycoprotein. Initial iodination of Tg produces monoiodotyrosine (MIT) and diiodotyrosine (DIT) while subsequent oxidation of MIT and DIT by TPO to radical species couples two residues of DIT, both still linked to the Tg, to produce thyroxine (T4) and couples one residue of MIT and one residue of DIT to produce triiodothyronine (T3). When thyroid hormones are needed, hormone-rich Tg is taken up into thyroid epithelial cells by endocytosis and digested by proteases which results in the release of T4 and T3 into the blood circulation through the action of their transporters. Chemicals potentially can suppress TPO-catalyzed iodination and/or coupling and in that way alter thyroid hormone homeostasis *in vivo*.

FTC-238-hrTPO cells are human thyroid carcinoma cells stably transfected with an expression clone coding for human recombinant (hr) TPO and can be used to prepare cell lysates containing the hrTPO enzyme. To evaluate potential interference with TPO-catalyzed iodination, FTC-238-hrTPO cell lysates were incubated with L-tyrosine, potassium iodide and H_2O_2 in the presence or absence of a test item. During incubation, TPO enzymatically converted L-tyrosine into MIT and formation of this metabolite was monitored by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as a direct measurement of TPO-catalyzed iodination. A small amount of MIT may also be formed non-enzymatically, its formation was assessed in separate incubations.

The Deviation and Study Plan are presented in Appendix 2.

Study Initiation Date: 30 Aug 2021

Experimental Start Date: 21 Sep 2021

Experimental Completion Date: 27 Oct 2021

4. MATERIALS AND METHODS

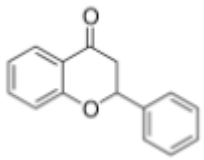
4.1. Test Materials

4.1.1. Test Item Characterization

Documentation of the identity, strength, purity, composition, and stability for the test items is available at the Test Facility. The characterization of the test items was conducted in an ISO quality environment. Certificates of Analysis were provided to the Test Facility and are presented in Appendix 3.

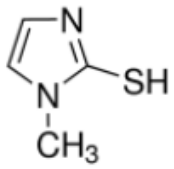
4.1.2. Test Items

4.1.2.1. Flavanone

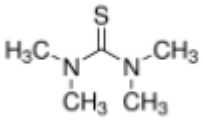
Identification:	Flavanone
Batch (Lot) Number:	MKCG6841
Expiry date:	19 June 2023
Physical Description:	White powder
Purity/Composition:	See Certificate of Analysis issued 19 June 2018
Storage Conditions:	At room temperature
Additional information	
Test Facility test item number:	212559/A
Purity/Composition correction factor:	No correction factor required
Chemical name (IUPAC, synonym or trade name):	2,3-Dihydroflavone (Flavanone)
CAS number:	487-26-3
Molecular structure:	
Molecular formula:	$C_{15}H_{12}O_2$
Molecular weight:	224.25 g/mol

4.1.2.2. Methimazole

Identification:	Methimazole
Batch (Lot) Number:	WXBC8588V
Expiry date:	19 October 2023
Physical Description:	Off-white powder
Purity/Composition:	See Certificate of Analysis issued 19 October 2018
Storage Conditions:	At room temperature
Additional information	

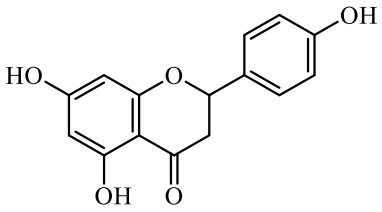
Test Facility test item number:	212556/A
Purity/Composition correction factor:	No correction factor required
Chemical name (IUPAC, synonym or trade name):	2-Mercapro-1-methylimidazole (MMI) 1-Methyl-2-imidazolethiol
CAS number:	60-56-0
Molecular structure:	
Molecular formula:	$C_4H_6N_2S$
Molecular weight:	114.17 g/mol

4.1.2.3. N,N,N,N-tetramethylthiourea

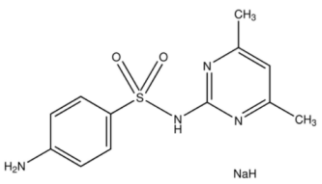
Identification:	N,N,N,N-tetramethylthiourea
Batch (Lot) Number:	SHBJ4707
Expiry date:	02 October 2022
Physical Description:	White powder
Purity/Composition:	See Certificate of Analysis issued 02 October 2017
Storage Conditions:	In refrigerator (2-8°C)
Additional information	
Test Facility test item number:	212555/A
Purity/Composition correction factor:	No correction factor required
Chemical name (IUPAC, synonym or trade name):	1,1,3,3-tetramethyl-2-thiourea
CAS number:	2782-91-4
Molecular structure:	
Molecular formula:	$C_5H_{12}N_2S$
Molecular weight:	132.23 g/mol

4.1.2.4. Naringenin

Identification:	Naringenin
Batch (Lot) Number:	A63107
Expiry date:	03 January 2024

Physical Description:	White solid
Purity/Composition:	See Certificate of Analysis issued 03 January 2019
Storage Conditions:	At room temperature
Additional information	
Test Facility test item number:	212557/A
Purity/Composition correction factor:	No correction factor required
Chemical name (IUPAC, synonym or trade name):	(2R)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one (4',5,7-Trihydroxyflavanone)
CAS number:	480-41-1
Molecular structure:	
Molecular formula:	$C_{15}H_{12}O_5$
Molecular weight:	272.3 g/mol

4.1.2.5. Sulfamethazine Sodium Salt

Identification:	Sulfamethazine sodium salt
Batch (Lot) Number:	WXBD0389V
Expiry date:	30 June 2023 (retest date)
Physical Description:	White powder
Purity/Composition:	100%
Storage Conditions:	In refrigerator (2-8°C)
Additional information	
Test Facility test item number:	RS739
Purity/Composition correction factor:	No correction factor required
CAS number:	1981-58-4
Molecular structure:	
Molecular formula:	$C_{12}H_{13}N_4NaO_2S$
Molecular weight:	300.31 g/mol

4.1.3. Control Items

4.1.3.1. Vehicle

Dimethyl sulfoxide (DMSO) was used as vehicle.

4.1.3.2. Substrate

L-tyrosine (CAS# 60-18-4, RS701) was used as substrate for the TPO-catalyzed iodination assay.

4.1.3.3. Metabolite

Monoiodotyrosine (MIT) (CAS# 70-78-0, AS2084) was the metabolite that was evaluated in the TPO-catalyzed iodination assay.

4.1.3.4. Internal Standard

Monoiodotyrosine-$^{13}C_6$ (MIT-$^{13}C_6$) (AS2083) was used as internal standard (IS) in the TPO-catalyzed iodination assay.

4.1.3.5. Negative Control

Bis-(2-ethylhexyl)-phthalate (DEHP, CAS# 117-81-7, RS493) was used as a negative control.

4.1.3.6. Reference Item

6-Propyl-2-thiouracil (PTU, CAS# 51-52-5, RS506) was used as a reference item.

4.2. Reserve Samples

For sulfamethazine, a reserve sample was collected and maintained under the appropriate storage conditions by the Test Facility.

For flavanone, methimazole, naringenin and TMTU, reserve samples were not collected and maintained due to the limited amounts available. The OECD GLP guidelines for short-term studies state that there is no need to retain a sample for short-term studies.

4.3. Test and Control Item and Internal Standard Inventory and Disposition

The test and control items and internal standard (IS) were received by the Test Facility for distribution as needed. Records of the receipt, distribution, and storage of test and control items were maintained. With the exception of the reserve sample, all unused Sponsor-supplied test item will be discarded or returned to the Sponsor after completion of the scheduled program of work. Records of the decisions made will be kept at the Test Facility.

4.4. Dose Formulation and Analysis

4.4.1. Preparation of Test Item Stock and Spiking Solutions

No correction for the purity/composition of the test items was performed.

Before performing the first TPO-catalyzed iodination experiment, a solubility test was performed for each test item to determine the appropriate concentration range to be tested (see Section 4.7.1.1).

Based on the solubility assessments, 10 mM stock solutions were prepared freshly in DMSO for each test item for each experiment on the day of use. In all cases, a clear colorless solution was formed for each test item.

For each test item and for each experiment, the test item stock solution was further diluted in DMSO to obtain eight 100× spiking solutions. The spiking solutions were further diluted in

the incubation mixtures. The final concentrations of each test item in the different TPO-catalyzed iodination experiments have been specified in the table below.

Text Table 2
Composition of Incubation Samples

Test Item	Final Concentrations used for First Experiment	Final Concentrations used for Remaining Experiments
Flavanone	10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM, 31.6 µM and 100 µM	10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM, 31.6 µM and 100 µM
TMTU		
Sulfamethazine		
Methimazole		3.16 nM, 10 nM, 31.6 nM, 100 nM, 316 nM, 1 µM, 3.16 µM and 10 µM
Naringenin		10 nM, 31.6 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM and 31.6 µM

Any residual volumes were discarded.

4.4.2. Preparation of Control Item Stock and Spiking Solutions

100 mM DEHP stock solutions were prepared in DMSO and stored in the freezer set to maintain -20°C for a maximum of one month.

The negative control inhibitor DEHP was tested at one concentration. For this purpose, the DEHP stock solution was further diluted in the incubation mixture. The final DEHP concentration in the TPO inhibition assay incubation mixture was 1 mM.

For each experiment, a 3.16 mM PTU stock solution was prepared freshly in DMSO on the day of use.

The PTU stock solution was further diluted in DMSO to obtain eight 100× spiking solutions. The spiking solutions were further diluted in the incubation mixtures. The final concentrations in the TPO-catalyzed iodination assay incubation mixtures were 1 nM, 10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM and 31.6 µM.

Any residual volumes were discarded.

4.4.3. Preparation of Internal Standard Solutions

No correction was made for the purity/composition of the IS.

A stock solution of the IS was prepared in 0.1 M HCl at a target concentration of 1 mg/mL. The stock solution was aliquoted in glass vials and stored in the freezer set to maintain -20°C.

The IS stock solution was diluted in methanol containing 300 µM sodium thiosulfate to obtain a solution of 3000 ng IS/mL (IS working solution). The IS working solution was prepared freshly on the day of use.

Any residual volumes were discarded.

4.5. Test System

Test system	FTC-238-hrTPO cell lysates.
Rationale	The FTC-238 human follicular thyroid carcinoma cell line was established from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old male. The cells are polymorphic showing flat polygonal to spindle-like morphologies. The FTC-238 cells are genetically modified to incorporate human recombinant TPO and a neomycin resistance gene.

	Prepared cell lysates of hematin-stimulated FTC-238-hrTPO cells contain active human thyoid peroxidase.
Source	The FTC-238-hrTPO cell line was provided by the study Sponsor EURL ECVAM, who obtained it from Charité.
Storage	FTC-238-hrTPO cell lysates were stored in an ultra-low freezer set to maintain -80°C.

4.6. Reagents

2-Propanol	LiChrosolv, Merck KGaA, Darmstadt, Germany
6-Propyl-2-thiouracil (PTU)	Batch BCBX0879, Sigma Aldrich Chemie GmbH, Schnelldorf, Germany
Acetonitrile (ACN)	ULC/MS, Biosolve B.V., Valkenswaard, The Netherlands
Bis-(2-ethylhexyl)-phthalate (DEHP)	Batch BCBX5578, Sigma Aldrich Chemie GmbH, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	SeccoSolv, Merck KGaA
Dipotassium hydrogen phosphate (K_2HPO_4)	p.a. Merck KGaA
Formic acid (FA)	99%, ULC/MS, Biosolve B.V.
Hydrochloric acid (HCl) 1N	TitriPUR, Merck KGaA
Hydrogen peroxide (H_2O_2) 30%	EMPROVE, Merck KGaA
L-tyrosine	Batch SLBW9140, Sigma Aldrich Chemie GmbH, Schnelldorf, Germany
Methanol (MeOH)	Absolute, ULC/MS, Biosolve B.V.
Milli-Q water (MQ)	Tap water purified by reversed osmosis and subsequently passed over activated carbon and ion exchange cartridges; Millipore, Bedford, MA, USA
Monoiodotyrosine (MIT)	Batch BCBZ6000, Sigma-Aldrich, Saint Louis, USA
Monoiodotyrosine-$^{13}C_6$ (MIT-$^{13}C_6$)	Batch 6-YSW-166-1, Toronto Research Chemicals, Toronto, Canada
Potassium dihydrogen phosphate (KH_2PO_4)	p.a. Merck KGaA
Potassium iodide (KI)	p.a. Merck KGaA
Sodium thiosulfate	Honeywell, Seelze, Germany

4.7. Experimental Design

Incubations were performed with FTC-238-hrTPO cell lysates and the test items to evaluate the possible suppressive effect of each test item on the TPO-catalyzed iodination by measuring the formation of MIT.

Five independent and valid runs were performed for each test item.

4.7.1. Main Study

4.7.1.1. Solubility Test

For each test item, a preliminary test was performed to determine whether the test item had any solubility problems, i.e., the presence of cloudiness or precipitate was evaluated. The test items were dissolved in DMSO to prepare a stock solution at an initial concentration of 100 mM. The presence of cloudiness or precipitate was evaluated by visual inspection and under the microscope. If necessary, a test item stock solution was further diluted (e.g., ½ log lower) to define the highest soluble concentration of test item in vehicle.

If a test item was soluble in vehicle, a 100-fold dilution of the stock solution was prepared in the incubation buffer and it was determined whether the test item had any solubility problems in the incubation buffer, i.e., the presence of cloudiness or precipitate was evaluated visually and using a light microscope. If necessary, the test item stock solution in vehicle was further diluted (e.g., ½ log lower) to define the highest soluble concentration of test item in the incubation buffer.

4.7.1.2. TPO-Catalyzed Iodination Assay

In the TPO-catalyzed iodination assay, incubations were performed with FTC-238-hrTPO cell lysates to determine the possible suppressive effect of the test items on the TPO-catalyzed iodination by measuring the formation of the metabolite MIT. Five independent and valid runs were performed for each test item. Concentrations of the test item used in a second and/or any subsequent experiments were adjusted, if necessary.

Incubation mixtures were prepared on ice by mixing phosphate buffer (0.1 M, pH 7.4), potassium iodide (final concentration 150 µM), L-tyrosine (final concentration 500 µM) FTC-238-hrTPO cell lysate (final concentration 100 µg/mL) and vehicle, test item or control item. After shaking, the samples were pre-incubated for 5 minutes at 37±1°C in a water bath and the reaction was started by the addition of 20 µL H_2O_2 (final concentration 250 µM). The total volume was 300 µL. Incubations were stopped after 15 minutes by transferring the reaction tubes to ice and addition of a half reaction volume of the 3000 ng/mL IS working solution. After vortex-mixing of the containers, the samples were kept on ice until centrifugation (2000 *g* for at least 5 minutes at 4°C) and prepared for MIT analysis by UPLC-MS/MS.

In total two incubation plates were prepared for each experiment. Vehicle controls, assay buffer controls (no vehicle controls), no peroxide controls, non-enzymatic iodination controls, the negative control DEHP at a single concentration (1 mM) and incubations with the appropriate test items were included on each incubation plate in triplicate. The first incubation plate also contained the reference item PTU which was tested at eight concentrations (1 nM, 10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM and 31.6 µM) in triplicate. The second incubation plate also contained the reference item PTU which was tested at the highest concentration (31.6 µM) in triplicate.

An overview of the incubations included for each independent experiment is presented in the table below.

Text Table 3
Composition of Incubation Samples

Constituent (final concentration)	No peroxide control	Vehicle control	No vehicle control	Non-enzymatic iodination control	Test item, reference item or negative control item incubations
Phosphate buffer (0.1 M, pH 7.4)	X	X	X	X	X
Tyrosine (500 µM)	X	X	X	X	X
Potassium Iodide (150 µM)	X	X	X	X	X
H_2O_2 (250 µM)	---	X	X	X	X
FTC-238-hrTPO cell lysate	X	X	X	---	X
Inactivated FTC-238-hrTPO cell lysate	---	---	---	X	---
Vehicle (1%)	X	X	---	X	---
Test item or PTU (in triplicate) and DEHP (in triplicate)	---	---	---	---	X
Number of incubations	6	6	6	6	153

4.7.2. Analysis of MIT

MIT concentrations in the samples were determined by UPLC-MS/MS using the method validated in Charles River Test Facility Study No. 20278296 (see Appendix 1).

4.7.2.1. MIT Stock and Spiking Solutions

Duplicate MIT stock solutions (stocks A and B) were prepared at a 10 mM concentration in 0.1 M HCl in glass vials. Stock solutions were prepared freshly on the day of use.

MIT stock solutions were diluted in 0.1 M potassium phosphate buffer pH 7.4 to obtain spiking solutions for the preparation of calibration standards and quality control (QC) samples. Spiking solutions were prepared freshly each experimental day as presented in the tables below.

Text Table 4
Preparation of MIT Spiking Solutions used for the Preparation of Calibration Standards

Code	Applied solution	Volume applied (µL)	Volume buffer added (µL) [1)]	Target concentration (µM)
Spike 0.0761 µM	Spike 0.352 µM	108	392	0.0761
Spike 0.163 µM	Spike 0.756 µM	108	392	0.163
Spike 0.352 µM	Spike 1.63 µM	108	392	0.352
Spike 0.756 µM	Spike 3.50 µM	108	392	0.756
Spike 1.63 µM	Spike 7.55 µM	108	392	1.63
Spike 3.50 µM	Spike 16.2 µM	108	392	3.50
Spike 7.55 µM	Spike 35.0 µM	108	392	7.55
Spike 16.2 µM	Spike 75.0 µM	108	392	16.2
Spike 35.0 µM	150 µM – B	116.5	383.5	35.0
Spike 75.0 µM	150 µM – A	300	300	75.0
150 µM – B	Stock B	15	985	150
150 µM – A	Stock A	15	985	150

[1)] The buffer consisted of 0.1 M potassium phosphate buffer pH 7.4.

Text Table 5
Preparation of MIT Spiking Solutions used for the Preparation of QC Samples

Code	Applied solution	Volume applied (µL)	Volume buffer added (µL) [1)]	Target concentration (µM)
Spike 15 µM	Spike 75 µM	200	800	0.150
Spike 75 µM	Spike 600 µM	125	875	0.750
Spike 600 µM	Spike 6 mM	100	900	NA
Spike 6 mM	Stock A	600	400	60.0

[1)] The buffer consisted of 0.1 M potassium phosphate buffer pH 7.4.
NA: Not applicable, the spiking solution was used as an intermediate solution only.

4.7.2.2. Calibration Standards

Ten calibration standards were prepared from MIT spiking solutions which were prepared from two MIT stock solutions (A and B as described in Section 4.7.2.1). An aliquot of the appropriate spiking solution was added to the IS working solution as described in the table below. Samples were vortex-mixed and prepared for UPLC-MS/MS analysis by diluting them 100-fold in phosphate buffer (0.1 M, pH 7.4). Calibration standards were prepared freshly on the first day of use.

Text Table 6
Preparation of Calibration Standards

Code	Applied solution	Volume Applied		Target concentration (µM) [2)]
		Spiking solution (µL)	IS Working Solution (µL)	
MIT 0.0 µM	Buffer [1)]	300	150	0
MIT 0.0761 µM	Spike 0.0761 µM	300	150	0.0761
MIT 0.163 µM	Spike 0.163 µM	300	150	0.163
MIT 0.352 µM	Spike 0.352 µM	300	150	0.352
MIT 0.756 µM	Spike 0.756 µM	300	150	0.756
MIT 1.63 µM	Spike 1.63 µM	300	150	1.63
MIT 3.50 µM	Spike 3.50 µM	300	150	3.50
MIT 7.55 µM	Spike 7.55 µM	300	150	7.55
MIT 16.2 µM	Spike 16.2 µM	300	150	16.2
MIT 35.0 µM	Spike 35.0 µM	300	150	35.0
MIT 75.0 µM	Spike 75.0 µM	300	150	75.0

[1)] The buffer consisted of 0.1 M potassium phosphate buffer pH 7.4.
[2)] Concentration in the sample before addition of the IS working solution (see Section 4.4.3).

The LLOQ (lower limit of quantification) was defined as 0.0761 µM while the ULOQ (upper limit of quantification) was defined as 75.0 µM when the acceptance criteria were met. Every value below LLOQ was not quantified and reported as < LLOQ.

4.7.2.3. Preparation of Quality Control (QC) Samples

Spiking solutions of the test item were applied to prepare quality control (QC)-0, -low (L), -middle (M), -high (H) samples. First, a blank matrix working solution was prepared by mixing the following components:

(1) 4631 µL 0.1 M potassium phosphate buffer pH 7.4
(2) 300 µL 10 mM L-Tyrosine dissolved in 0.1 M HCl
(3) 9 µL 100 mM potassium iodide prepared in buffer (1)
(4) 600 µL 500 µg/mL of heat-inactivated FTC-238-hrTPO cell lysate solution in buffer (1)

An aliquot of the appropriate spiking solution was added to the blank matrix working solution as described in the table below. The QC samples were prepared freshly on the first day of use.

Text Table 7
Preparation of the Test Item QC-L QC-M and QC-H Samples

Code	Applied solution	Volume Applied			Target concentration (µM) [2)]
		Spiking solution (µL)	Blank Matrix Working Solution (µL)	IS Working Solution (µL)	
QC-0	Buffer [1)]	3	277	150	0
QC-L	Spike 15 µM	3	277	150	0.150
QC-M	Spike 75 µM	3	277	150	0.750
QC-H	Spike 6 mM	3	277	150	60.0

[1)] The buffer consisted of 0.1 M phosphate buffer pH 7.4.
[2)] Concentration in the sample before addition of the IS working solution (see Section 4.4.3).

QC-0, QC-L, QC-M and QC-H samples were prepared in polypropylene tubes in quadruplicate as described in the table above and were vortex-mixed after which 20 µL H_2O_2 was added to each tube. After vortex-mixing of the containers one by one, the samples were kept on ice until centrifugation (2000 *g* for at least 5 minutes at 4°C) and prepared for UPLC-MS/MS analysis by diluting them 100-fold in potassium phosphate buffer (0.1 M, pH 7.4).

4.7.2.4. UPLC-MS/MS Analysis

MIT and IS peak areas in the samples were measured by UPLC-MS/MS using the following system:

- Acquity UPLC I-Class system (Waters, Milford, MA, USA)
- Xevo TQ-(X)S mass spectrometer (Waters)

Data were acquired and interpreted with MassLynx software (Waters).

5. ACCEPTABILITY CRITERIA

5.1. Sample Analysis

An UPLC-MS/MS analytical run was considered acceptable if the criteria for the calibration curve and the QC samples were met.

5.1.1. Calibration Curve

The response of the calibration standards was correlated with the nominal MIT concentration of the calibration solutions using regression analysis with a $1/x^2$ weighting factor. Calibration curves with back calculated accuracies within the criterion range of 80-120% of the nominal concentration for the lowest calibration standard and 85-115% of the nominal concentration for the remaining calibration standards were accepted.

When a back calculated accuracy (once established) did not comply with the criterion range, the response of the calibration standard with the highest deviation was rejected and the calibration curve was re-evaluated. Calibration curves were accepted when ≥ 75% of the calibration standards fulfilled the acceptance criteria.

Zero was not part of the calibrated range. Blank samples were not taken into account in the fitting procedure.

5.1.2. QC Samples

The analytical method was considered applicable for the quantitative analysis of MIT in the samples when the accuracy of the QC samples was in the criterion range of 85-115%. Results outside the criterion range were discarded as long as per run 2/3 of the QC samples were accepted with ≥ 50% of each level.

5.2. TPO-Catalyzed Iodination Assay

An independent TPO-catalyzed iodination experiment was considered acceptable if the following criteria were met:

- The final curve for the reference item PTU was composed of a minimum of six concentrations obtained from the average of three replicates after excluding samples on the basis of insolubility, operator errors or other information.
- The final curve for a test item was composed of a minimum of six concentrations obtained from the average of three replicates after excluding samples on the basis of insolubility, operator errors or other information.
- The percent TPO-catalyzed iodination of the lowest test item concentration was within the range of 80%-120% when compared to the average activity in the vehicle control samples.
- A complete sigmoidal curve for the reference item PTU was obtained.
- The calculated IC_{50} for PTU was within the range of 5×10^{-7}-5×10^{-6} M.
- The percent TPO-catalyzed iodination compared to the vehicle control for the negative control DEHP was > 80%.
- The percent TPO-catalyzed iodination in each of the individual no-peroxide control samples was < 1% when compared to the average activity in the vehicle control samples.
- The mean percent TPO-catalyzed iodination of the no-vehicle controls was within the range of 80%-120% when compared to the average TPO-catalyzed iodination in the vehicle control samples.

5.3. Data Interpretation Criteria

For each run, a test item was considered negative when the percentage of TPO-catalyzed iodination compared to the average activity in the vehicle control samples was not less than 80% for any concentration.

For each run, a test item was considered positive when the percent of TPO-catalyzed iodination compared to the average activity in the vehicle control samples was less or equal to 80% for at least one concentration and was showing a dose-dependent effect.

A run was considered inconclusive in all other cases.

If (one of) the acceptability criteria were not met and the Study Director decided that this had a critical effect on the study, the test was rejected and repeated.

6. ANALYSIS

6.1. MIT Analysis

Response (R)

Peak area of the analyte × (IS Conc./ IS peak area) [units]

Calibration curve

$$R = a \times C_N + b$$

where:

a = linear regression factor

b = intercept

C_N = nominal concentration

Regression analysis was performed using the least squares method.

Analyzed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \text{ [µM]}$$

Accuracy of analytical QC samples

$$\frac{C_A - C_B}{C_N} \times 100 \text{ [\%]}$$

where:

C_B = analyzed concentration in QC-0 sample

6.2. TPO-Catalyzed Iodination

The percent of TPO-catalyzed iodination compared to the average TPO-catalyzed iodination in the vehicle control samples (= full activity) was calculated for each individual sample (vehicle control, no vehicle control, no peroxide control, DEHP, PTU and test item samples) using the following equation:

$$\%\text{TPO catalyzed iodination} = \frac{C_A \text{ in sample}}{\text{Average } C_A \text{ of vehicle control samples}} \times 100\%$$

If applicable, the IC_{50} value was calculated by plotting the percentage of control activity versus the logarithm of the concentration fitted by the Hill curve model (variable slope, 4 parameters) using GraphPad Prism (GraphPad Software 8.4.2, San Diego, USA) and the following equation:

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left(1 + 10^{((\text{Log}IC_{50} - x) * \text{HillSlope})}\right)}$$

In which the variables were defined as follows:

Y = Percent of the control activity
X = Logarithm (base 10) of the concentration
Top = Top of the curve in same units as Y
Bottom = Bottom of the curve in same units as Y
Log IC_{50} = Logarithm of concentration at which 50% of maximum response is observed
HillSlope = Slope factor of the Hill curve

7. COMPUTERIZED SYSTEMS

Computerized systems used in the study are listed below. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 8
Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
Share Document Management System	1.0	Reporting
M-Files®	21.1	Reporting and collection of 21 CFR Part 11 compliant signature
MassLynx	4.2	System control, data acquisition and integration
REES Centron	SQL 2.0	Temperature monitoring

8. RETENTION AND DISPOSITION OF RECORDS AND SAMPLES

All study-specific raw data, documentation, Study Plan, and Final Report from this study were archived at the Test Facility at finalization of the report. At least two years after issue of the Final Report, the Sponsor will be contacted.

Electronic data generated by the Test Facility were archived as noted above, except that files stored on M-Files® (Study Plan and reporting files) were archived at the Charles River Laboratories facility location in Wilmington, Massachusetts, USA.

A verified copy of the applicable study-specific raw data was sent to the Sponsor at the following location:

European Commission (DG-JRC)
Directorate F - Health, Consumers and Reference Materials
Unit F3 - Chemical Safety and Alternative Methods / The European Union Reference Laboratory for Alternatives to Animal Testing
(EURL ECVAM)
Via E. Fermi, 2749. TP126
I-21027 Ispra (VA)
Italy

Disposition of residual/retained analytical samples was as described in the table below.

Text Table 9
Disposition of Residual/Retained Samples

Sample Type	Disposition
Analytical (and test article used in analysis)	Discarded

9. RESULTS

9.1. Solubility Assessment

Methimazole, sulfamethazine and TMTU were all soluble in DMSO at a concentration of 100 mM and a 100-fold dilution of these 100 mM stock solutions in incubation buffer resulted in the formation of clear solutions.

Naringenin was not soluble in DMSO at a concentration of 100 mM but was soluble in DMSO at a concentration of 31.6 mM. A 100-fold dilution of this 31.6 mM stock solution in incubation buffer resulted in the formation of a clear solution.

Flavanone was soluble in DMSO at a concentration of 100 mM, however, a 100-fold dilution of this 100 mM stock solution in incubation buffer resulted in precipitation of the test item. A similar effect was observed for a test item stock concentration of 31.6 mM. A 100-fold dilution of a 10 mM solution of the test item in incubation buffer resulted in formation of a clear solution.

Based on the solubility assessments, a 10 mM stock solution was used for each test item as highest test item spiking concentration in the TPO inhibition assay experiment (final concentration in incubation mixture: 100 µM).

9.2. TPO-Catalyzed Iodination Assay

In total six individual experiments were performed to evaluate the potential of the different test items to suppress TPO-catalyzed iodination. The results of the experiments performed on 23 Sep 2021 (experiment 1), 28 Sep 2021 (experiment 2), 30 Sep 2021 (experiment 3), 07 Oct 2021 (experiment 5) and 26 Oct 2021 (experiment 6) were considered valid and have been reported below.

The results of the experiment performed on 05 Oct 2021 (experiment 4) were rejected as the pH of the incubation buffer that was used was too high (7.4 instead of 7.8). The results of this experiment have been included in the raw data files of the study but will not be reported.

9.2.1. Results for the Calibration Curves and QC Samples

The analytical run history is presented in Table 1. The results for the calibration curves and QC samples obtained for the different experiments are presented in Table 2 and Table 3, respectively.

A linear relationship was observed between response and concentration for MIT in the range of 0.0761 – 75.0 µM for each of the curves. As the back calculated accuracies of all data points were in the range of 85-115% for all calibration standards for each of the curves, all curves were accepted.

The mean accuracies of all QC samples at each QC level were within 85-115% and as a result of that, all analytical runs were accepted.

9.2.2. Results for Controls

The results obtained for the vehicle, no vehicle, non-enzymatic iodination, no peroxide and negative (DEHP) controls are presented for each of the five valid experiments in Table 4 up to Table 8.

For each of the experiments, the percent TPO-catalyzed iodination compared to the vehicle control was ≥ 92% for the negative control DEHP which was well above the acceptance criterion of > 80%.

For each of the experiments, the mean MIT concentrations of the no-peroxide controls were always below the LOQ (0.0761 µM) and were therefore considered background. These results confirmed that MIT formation in the incubations is dependent on the presence of H_2O_2.

For each of the experiments, the mean percent TPO-catalyzed iodination of the no-vehicle controls when compared to the average TPO-catalyzed iodination in the vehicle control samples varied between 98% and 111% and thus was within the range of 80%-120%.

As all acceptance criteria for the controls were met, the TPO-catalyzed iodination experiments were considered valid.

9.2.3. Results for the Reference Item PTU

In each TPO-catalyzed iodination experiment, the reference item PTU was included at eight concentrations in triplicate. Detailed results obtained for PTU are presented in Table 9 up to Table 13. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 1 and Figure 2, respectively. The curve fit parameters, log IC_{50} and IC_{50} obtained for PTU are presented in the table below.

Text Table 10
Curve Fit Parameters, Log IC_{50} and IC_{50} obtained for PTU

Parameter	Exp 1	Exp 2	Exp 3	Exp 5	Exp 6	Average
Bottom (%)	-0.4675	1.538	-1.187	1.693	2.430	-0.7556
Top (%)	104.7	105.7	105.4	101.6	114.5	106.6
Log IC_{50} (M)	-5.713	-5.843	-5.716	-5.773	-5.918	-5.794
HillSlope	-2.194	-3.097	-1.907	-3.041	-3.175	-2.412
IC_{50} (µM)	1.938	1.434	1.923	1.686	1.207	1.606
Values	24	24	23	23	24	118

For each of the experiments, the curves for the reference item PTU were sigmoidal and were composed of a minimum of six concentrations. The reference item PTU suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 1.21 µM up to 1.94 µM which was within the acceptance range of 5×10^{-7}-5×10^{-6} M. The averaged IC_{50} value for all experiments was found to be 1.61 µM.

As all acceptance criteria for the reference item PTU were met, the TPO-catalyzed iodination experiments were considered valid.

9.2.4. Results for the Test Items

9.2.4.1. TMTU

TMTU was included at eight concentrations in triplicate in each TPO-catalyzed iodination experiment. Detailed results obtained for TMTU are presented in Table 14 up to Table 18. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 3 and Figure 4, respectively. The curve fit parameters, log IC_{50} and IC_{50} obtained for TMTU are presented in the table below.

Text Table 11
Curve Fit Parameters, Log IC_{50} and IC_{50} obtained for TMTU

Parameter	Exp 1	Exp 2	Exp 3	Exp 5	Exp 6	Average
Bottom (%)	0.4678	1.732	-0.02409	1.575	3.124	-0.4361
Top (%)	101.0	101.0	105.2	105.9	111.6	105.1
Log IC_{50} (M)	-5.763	-5.846	-5.775	-5.802	-5.945	-5.821
HillSlope	-2.356	-2.959	-2.036	-2.460	-2.810	-2.323
IC_{50} (µM)	1.725	1.424	1.679	1.577	1.134	1.509
Values	24	24	24	24	24	120

For each of the experiments, the curves for the test item TMTU were sigmoidal and were composed of a minimum of six concentrations. The mean percent TPO-catalyzed iodination of the lowest test item concentration varied between 103% and 113% and thus was within the acceptance range of 80%-120% when compared to the average activity in the vehicle control samples.

Based on the outcome of the five valid TPO-catalyzed iodination experiments, it was concluded that the test item TMTU was positive and suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 1.13 µM up to 1.73 µM. The averaged IC_{50} value for all experiments was found to be 1.51 µM.

9.2.4.2. Methimazole

Methimazole was included at eight concentrations in triplicate in each TPO-catalyzed iodination experiment. Detailed results obtained for methimazole are presented in Table 19 up to Table 23. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 5 and Figure 6, respectively. The curve fit parameters, log IC_{50} and IC_{50} obtained for Methimazole are presented in the table below.

Text Table 12
Curve Fit Parameters, Log IC_{50} and IC_{50} obtained for Methimazole

Parameter	Exp 1	Exp 2	Exp 3	Exp 5	Exp 6	Average*
Bottom (%)	0.9488	1.160	0.6231	2.000	1.743	1.126
Top (%)	93.84	102.0	102.7	100.8	111.4	1.305
Log IC_{50} (M)	-6.406	-6.438	-6.406	-6.943	-6.438	-6.415
HillSlope	-6.301	-3.596	-4.782	-22.24	-2.601	-3.789
IC_{50} (µM)	0.3929	0.3650	0.3923	0.1140	0.3652	0.3842
Values	24	24	23	20	24	95

* For the construction of the averaged dose response curve, the data from experiment 5 have been excluded as the individual dose response curve for this experiment differed significantly from the other curves.

For each of the experiments, the curves for the test item methimazole were sigmoidal and were composed of a minimum of six concentrations. The mean percent TPO-catalyzed iodination of the lowest test item concentration varied between 97% and 116% and thus was within the acceptance range of 80%-120% when compared to the average activity in the vehicle control samples.

The results obtained during experiment 5 differed significantly from the results obtained for the other four experiments and therefore have been excluded from the determination of the IC_{50} range and calculation of the average IC_{50} values.

Based on the outcome of the five valid TPO-catalyzed iodination experiments, it was concluded that the test item methimazole was positive and suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 365 nM up to 393 nM. The averaged IC_{50} value for all experiments was found to be 384 nM.

9.2.4.3. Naringenin

Naringenin was included at eight concentrations in triplicate in each TPO-catalyzed iodination experiment. Detailed results obtained for naringenin are presented in Table 24 up to Table 28. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 7 and Figure 8, respectively. The curve fit parameters, log IC_{50} and IC_{50} obtained for naringenin are presented in the table below.

Text Table 13
Curve Fit Parameters, Log IC_{50} and IC_{50} obtained for Naringenin

Parameter	Exp 1	Exp 2	Exp 3	Exp 5	Exp 6	Average
Bottom (%)	-0.8977	-0.2776	-1.417	-0.4922	1.103	-1.663
Top (%)	111.3	108.2	103.2	96.36	108.4	105.1
Log IC_{50} (M)	-6.403	-6.388	-6.275	-6.254	-6.504	-6.355
HillSlope	-1.304	-1.392	-1.492	-1.698	-1.496	-1.421
IC_{50} (µM)	0.3954	0.4089	0.5313	0.5569	0.3131	0.4420
Values	24	24	24	24	24	120

For each of the experiments, the curves for the test item naringenin were sigmoidal and were composed of a minimum of six concentrations. The mean percent TPO-catalyzed iodination of the lowest test item concentration varied between 99% and 113% and thus was within the acceptance range of 80%-120% when compared to the average activity in the vehicle control samples.

Based on the outcome of the five valid TPO-catalyzed iodination experiments, it was concluded that the test item naringenin was positive and suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 313 nM up to 557 nM. The averaged IC_{50} value for all experiments was found to be 442 nM.

9.2.4.4. Flavanone

Flavanone was included at eight concentrations in triplicate in each TPO-catalyzed iodination experiment. Detailed results obtained for flavanone are presented in Table 29 up to Table 33. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 9 and Figure 10, respectively.

For each of the experiments, the mean percentage of TPO-catalyzed iodination compared to the average activity in the corresponding vehicle control samples was not less than 80% for any of the concentrations evaluated.

Based on the outcome of the five valid TPO-catalyzed iodination experiments, it was concluded that the test item flavanone was negative and did not suppressed the TPO-catalyzed iodination.

9.2.4.5. Sulfamethazine

Sulfamethazine was included at eight concentrations in triplicate in each TPO-catalyzed iodination experiment. Detailed results obtained for sulfamethazine are presented in Table 34 up to Table 38. The individual dose response curves for each experiment and the averaged dose response curve based on all experiments are presented in Figure 11 and Figure 12, respectively. The curve fit parameters, log IC_{50} and IC_{50} obtained for Sulfamethazine are presented in the table below.

Text Table 14
Curve Fit Parameters, Log IC_{50} and IC_{50} obtained for Sulfamethazine

Parameter	Exp 1	Exp 2	Exp 3	Exp 5	Exp 6	Average
Bottom (%)	-24.72	-8.159	-6.934	-1.813	-15.70	-9.762
Top (%)	107.1	105.3	109.9	97.38	120.1	106.7
Log IC_{50} (M)	-4.830	-4.920	-4.924	-4.954	-5.064	-4.942
HillSlope	-0.7117	-0.9932	-1.037	-1.345	-0.7744	-0.9569
IC_{50} (µM)	14.80	12.02	11.92	11.11	8.626	11.43
Values	23	23	24	23	16	109

For each of the experiments, the curves for the test item sulfamethazine were sigmoidal and were composed of a minimum of six concentrations. The mean percent TPO-catalyzed

iodination of the lowest test item concentration for experiments 1, 2, 3 and 5 varied between 99% and 113% whereas the mean percent TPO-catalyzed iodination of the lowest test item in experiment 6 was 123%. The latter value was slightly outside the acceptance range of 80%-120%, however, results for this experiment were accepted as the overall curve shape was similar to that of the curves obtained for the other experiments.

Based on the outcome of the five valid TPO-catalyzed iodination experiments, it was concluded that the test item sulfamethazine was positive and suppressed the TPO-catalyzed iodination in a dose-dependent manner with IC_{50} values ranging from 8.63 µM up to 14.8 µM. The averaged IC_{50} value for all experiments was found to be 11.4 µM.

10. CONCLUSION

Five valid TPO inhibition experiments were performed. The test items TMTU, methimazole, naringenin and sulfamethazine were found to be positive and suppressed the human TPO-catalyzed iodination in a dose-dependent manner. The test item flavanone was found to be negative and did not suppress the human recombinant TPO-catalyzed iodination. By performing these experiments, it was demonstrated that the developed method to determine the suppression of human recombinant TPO-catalyzed iodination *in vitro* was robust and reliable.

11. REFERENCES

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3. Price, R.J., Burch, R., Chatham, L.R., Higgins, L.G., Currie, R.A., Lake, B.G., An assay for screening xenobiotics for inhibition of rat thyroid gland peroxidase activity. Xenobiotica, DOI: 10.1080/00498254.2019.1629044.

12. LIST OF ABBREVIATIONS

ACN	Acetonitrile
DEHP	Di-(2-ethylhexyl)phthalate
DMSO	Dimethyl sulfoxide
GLP	Good laboratory practice
HCl	Hydrogen chloride
hr	Human recombinant
IC_{50}	Inhibition at 50% of vehicle control
IS	Internal standard
KI	Potassium iodide
LLOQ	Lower limit of quantification
MeOH	Methanol
MIT	Mono-iodotyrosine
MIT-$^{13}C_6$	Mono-iodotyrosine-$^{13}C_6$
MQ	Milli-Q water
NA	Not applicable
PTU	6-propyl-2-thiouracil
QAU	Quality assurance unit
r^2	Determination coefficient
RTM	Rat thyroid microsomes
SD	Standard deviation
TPO	Thyroid peroxidase
TMTU	N,N,N,N-tetramethylthiourea
ULOQ	Upper limit of quantification
UPLC-MS/MS	Ultraperformance liquid chromatography tandem mass spectrometry

Figure 1
Individual Dose Response Curves for the Reference Control PTU obtained in the five valid TPO-Catalyzed Iodination Experiments

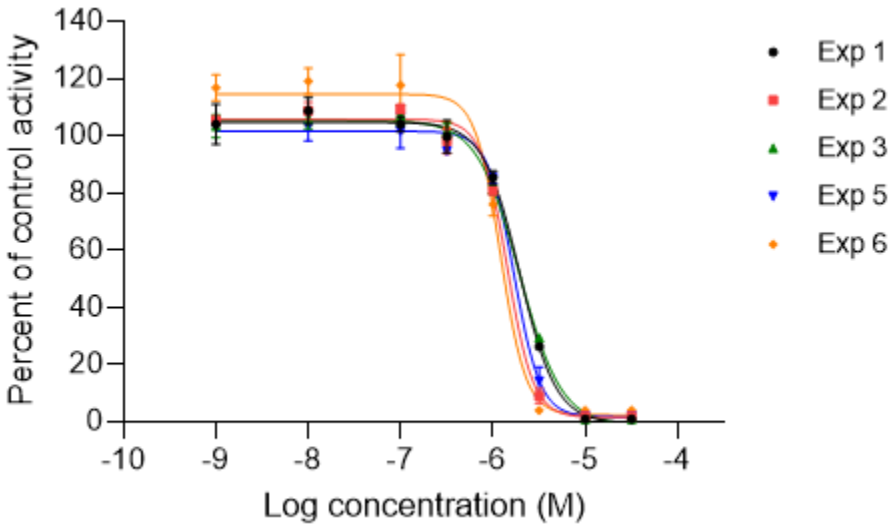


Figure 2
Averaged Dose Response Curve for the Reference Control PTU

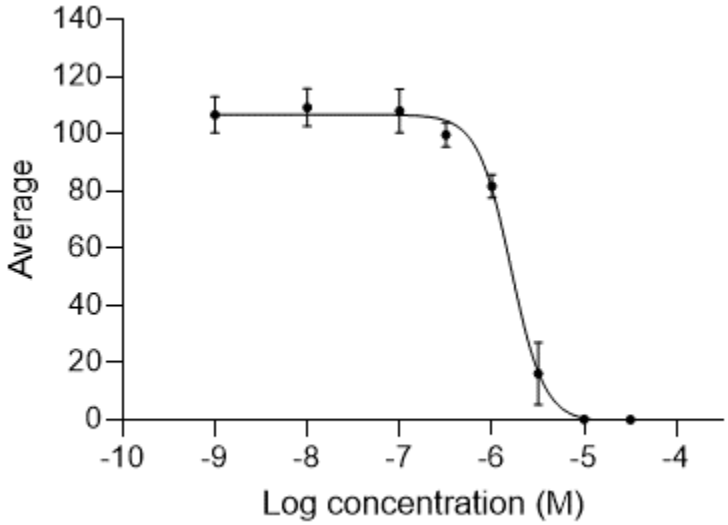


Figure 3
Individual Dose Response Curves for the Test Item TMTU obtained in the five valid TPO-Catalyzed Iodination Experiments

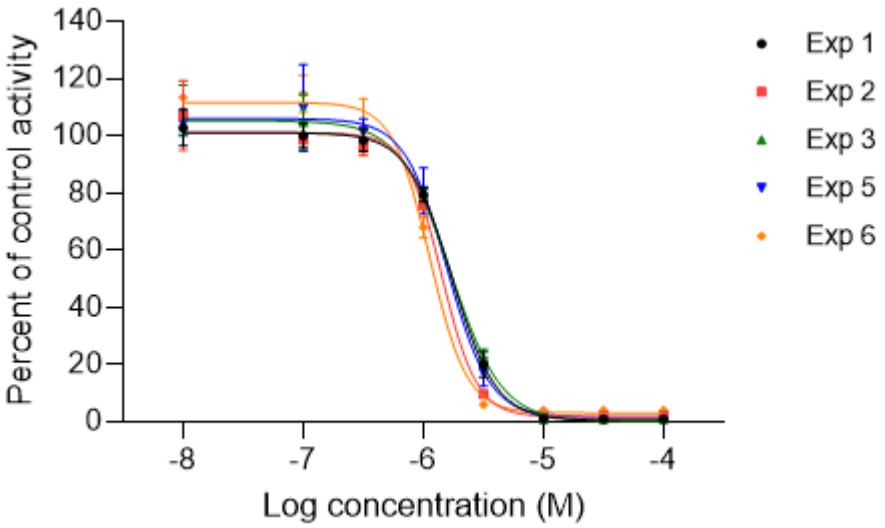


Figure 4
Averaged Dose Response Curve for the Test Item TMTU

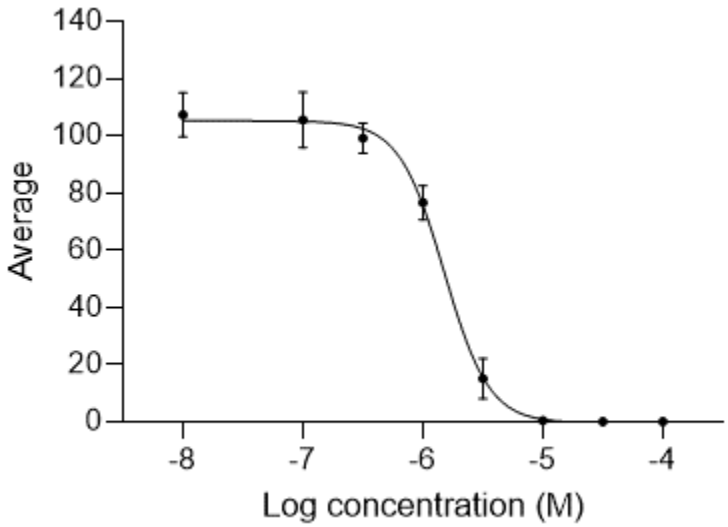


Figure 5
Individual Dose Response Curves for the Test Item Methimazole obtained in the five valid TPO-Catalyzed Iodination Experiments

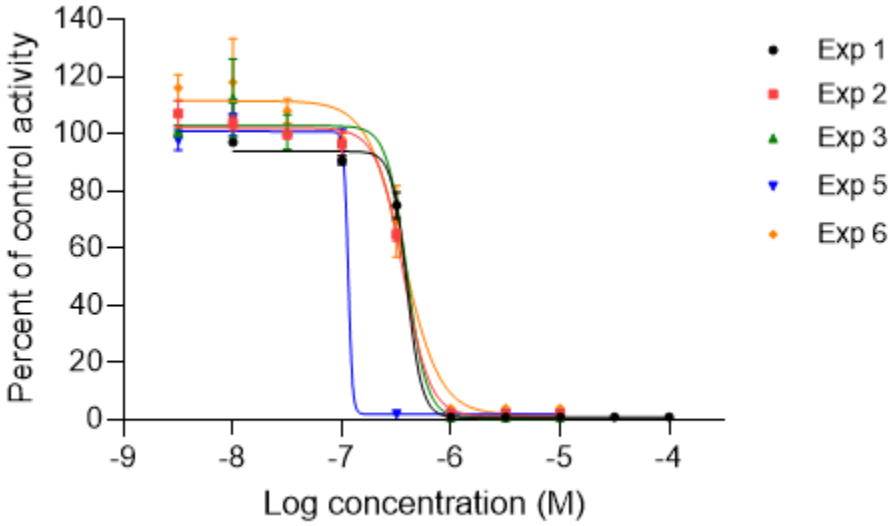
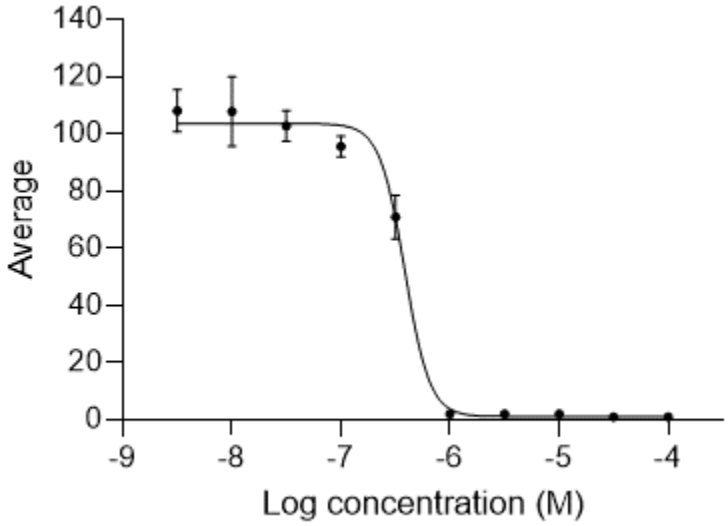


Figure 6
Averaged Dose Response Curve for the Test Item Methimazole



For the construction of the averaged dose response curve, the data from experiment 5 have been excluded as the individual dose response curve for this experiment differed significantly from the other curves.

Figure 7
Individual Dose Response Curves for the Test Item Naringenin obtained in the five valid TPO-Catalyzed Iodination Experiments

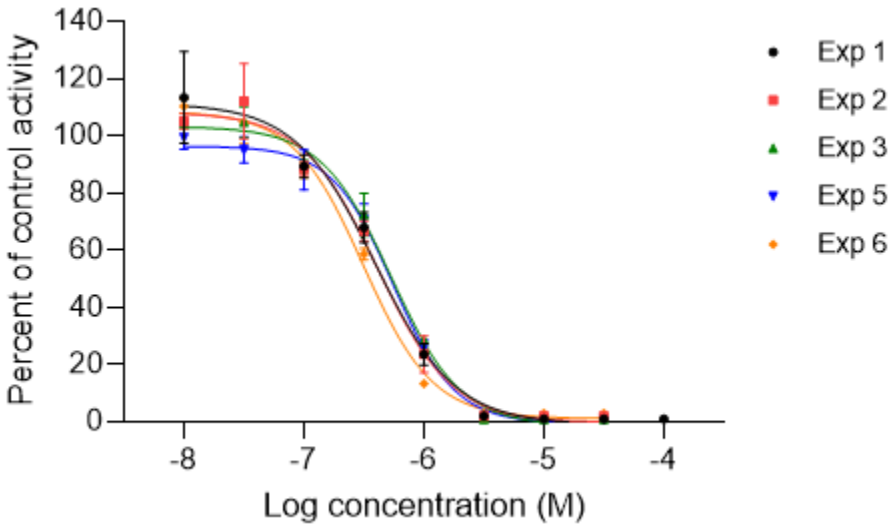


Figure 8
Averaged Dose Response Curve for the Test Item Naringenin

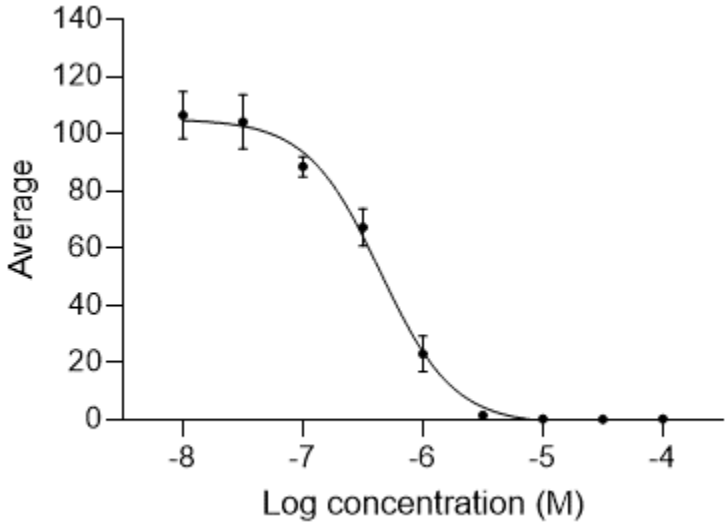


Figure 9
Individual Dose Response Curves for the Test Item Flavanone obtained in the five valid TPO-Catalyzed Iodination Experiments

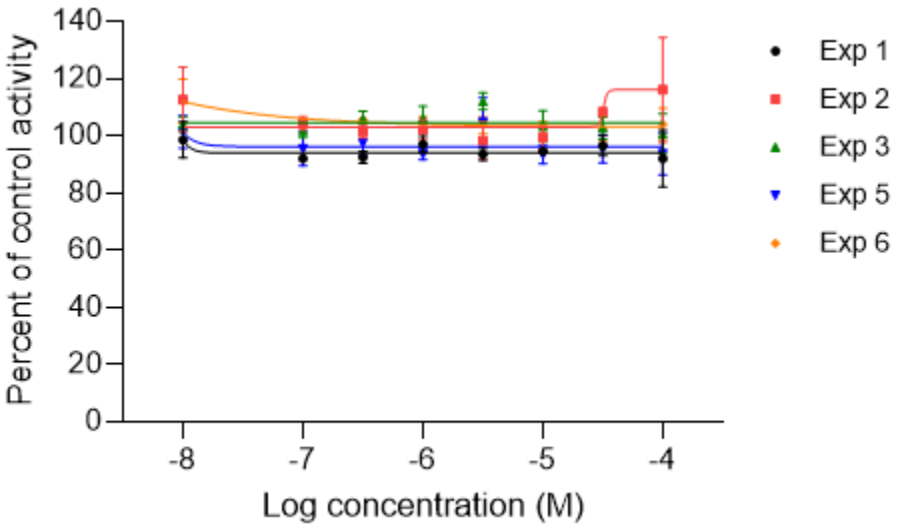


Figure 10
Averaged Dose Response Curve for the Test Item Flavanone

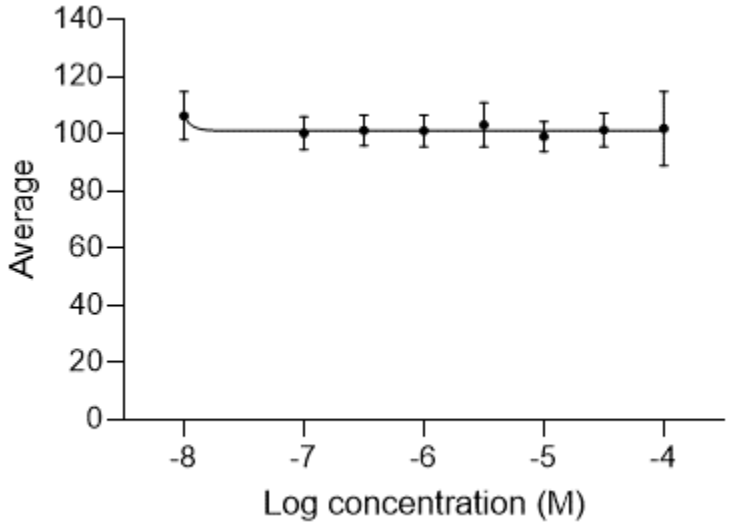


Figure 11
Individual Dose Response Curves for the Test Item Sulfamethazine obtained in the five valid TPO-Catalyzed Iodination Experiments

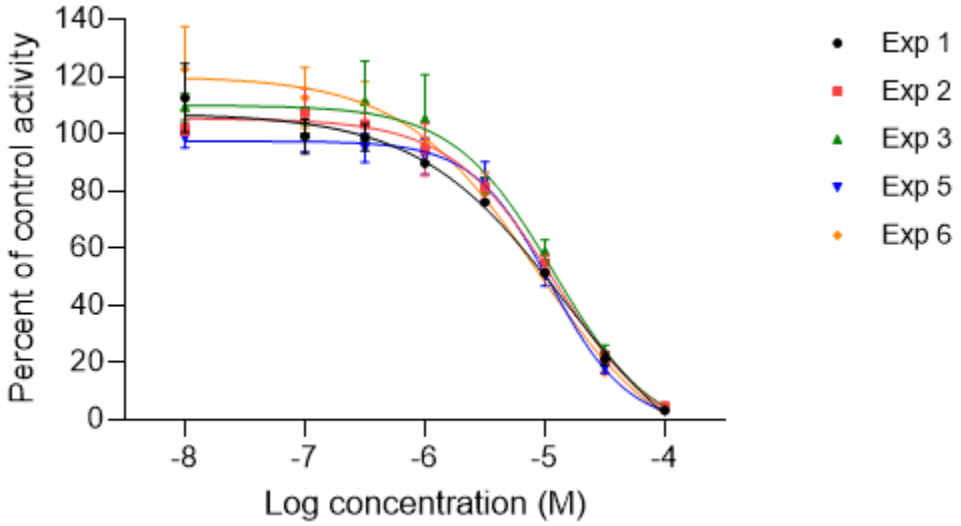


Figure 12
Averaged Dose Response Curve for the Test Item Sulfamethazine

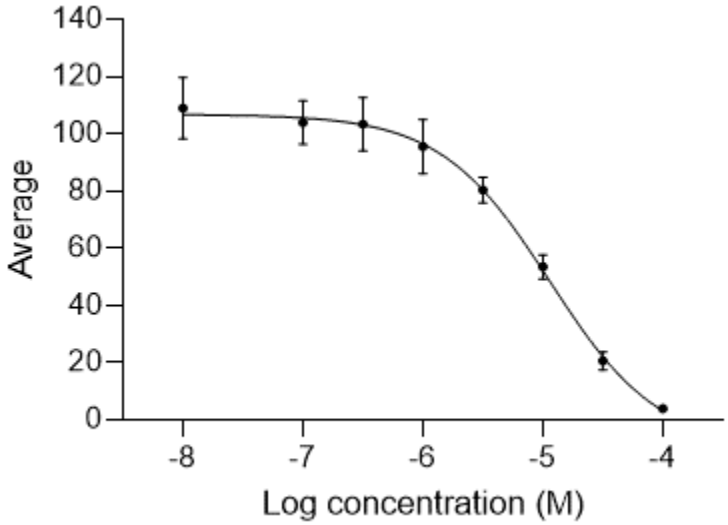


Table 1
Analytical Run History

Analytical Run	Run No.	Experimental Date [dd/Mmm/yyyy]	Remarks
20309164 TPO Main 1 23Sep2021	MIT_1	23/Sep/2021	Calibration standards and QCs prepared freshly
20309164 TPO Main 2 28Sep2021	MIT_2	28/Sep/2021	Calibration standards and QCs prepared freshly
20309164 TPO Main 3 30Sep2021	MIT_3	30/Sep/2021	Calibration standards and QCs prepared on 28 Sep 2021
20309164 TPO Main 4 05Oct 2021	MIT_4	05/Oct/2021	Calibration standards and QCs prepared freshly*, samples were reanalyzed (incorrect MS tune file used).
20309164 TPO Main 4 05Oct2021_Reinject	MIT_5	05/Oct/2021	Reanalysis of MIT_4*
20309164 TPO Main 5 07Oct2021	MIT_6	07/Oct/2021	Calibration standards and QCs prepared on 07 Oct 2021*
20309164 TPO Main 6 26Oct2021	MIT_7	26/Oct/2021	Calibration standards and QCs prepared freshly

* Calibration standards and QC samples were prepared in 0.1 M potassium phosphate buffer pH 7.8 (instead of 7.4). A deviation has been prepared for this.

Table 2
Individual Data and Statistical Parameters of the MIT Calibration Curves

Run No.	Range (µM)	Accepted points	Slope	Intercept	r^2
MIT_1	0.0761 – 75.0	16/16	2.33×10^{-1}	1.48×10^{-3}	0.998
MIT_2	0.0761 – 75.0	16/16	2.41×10^{-1}	2.34×10^{-3}	0.997
MIT_3	0.0761 – 75.0	16/16	2.42×10^{-1}	2.00×10^{-3}	0.997
MIT_4	0.0761 – 75.0	Samples were reanalyzed, data was not processed			
MIT_5	0.0761 – 75.0	16/16	2.86×10^{-1}	2.44×10^{-3}	0.998
MIT_6	0.0761 – 75.0	16/16	2.85×10^{-1}	2.04×10^{-3}	0.997
MIT_7	0.0761 – 75.0	16/16	2.38×10^{-1}	1.31×10^{-3}	0.992

Table 3
Accuracy and Repeatability of MIT QC Samples

Run No.	Range (µM)	Mean Accuracy (%)		
		QC-L (Accepted points)	QC-M (Accepted points)	QC-H (Accepted points)
MIT_1	0.0761 – 75.0	103 (4/4)	99 (4/4)	94 (4/4)
MIT_2	0.0761 – 75.0	94 (4/4)	99 (4/4)	97 (4/4)
MIT_3	0.0761 – 75.0	95 (4/4)	99 (4/4)	96 (4/4)
MIT_4	0.0761 – 75.0	Samples were reanalyzed, data was not processed		
MIT_5	0.0761 – 75.0	91 (4/4)	100 (4/4)	98 (4/4)
MIT_6	0.0761 – 75.0	93 (4/4)	98 (4/4)	97 (4/4)
MIT_7	0.0761 – 75.0	92 (4/4)	91 (4/4)	91 (4/4)

Table 4
Results obtained for Controls in TPO-Catalyzed Iodination Experiment 1

Sample Type	MIT Concentration (µM)				% Compared to Vehicle Control
	Replicate			Mean	Mean
	A	B	C		
Plate 1					
Negative control (1 mM DEHP)	5.90	5.76	5.82	5.83	97
Vehicle control	6.18	5.77	6.09	6.01	100
No vehicle control	6.47	5.77	6.06	6.10	101
Non-enzymatic iodination control	0.0492*	0.0427*	0.0511*	0.0761	1
No peroxide control	0.0021*	0.0031*	0.0024*	0.0761	1
Plate 2					
Negative control (1 mM DEHP)	5.95	5.68	5.47	5.70	97
Vehicle control	5.68	6.03	5.92	5.88	100
No vehicle control	6.04	6.03	5.85	5.97	102
Non-enzymatic iodination control	0.0478*	0.0460*	0.0555*	0.0761	1
No peroxide control	0.111	0.0620*	0.0625*	0.0877	1

The activity of the vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 5
Results obtained for Controls in TPO-Catalyzed Iodination Experiment 2

Sample Type	MIT Concentration (µM)				% Compared to Vehicle Control
	Replicate			Mean	Mean
	A	B	C		
Plate 1					
Negative control (1 mM DEHP)	4.36	4.35	4.33	4.34	98
Vehicle control	4.52	4.37	4.43	4.44	100
No vehicle control	4.73	4.77	4.65	4.72	106
Non-enzymatic iodination control	0.0418*	0.0465*	0.0534*	0.0761	2
No peroxide control	0.0000*	0.0000*	0.0006*	0.0761	2
Plate 2					
Negative control (1 mM DEHP)	4.34	3.50	3.75	3.86	97
Vehicle control	4.06	4.18	3.68	3.97	100
No vehicle control	4.36	4.47	4.42	4.42	111
Non-enzymatic iodination control	0.121	0.118	0.151	0.130	3
No peroxide control	0.0000*	0.0000*	0.0000*	0.0761	2

The activity of the vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 6
Results obtained for Controls in TPO-Catalyzed Iodination Experiment 3

Sample Type	MIT Concentration (µM)				% Compared to Vehicle Control
	Replicate			Mean	Mean
	A	B	C		
Plate 1					
Negative control (1 mM DEHP)	5.30	5.00	5.96	5.42	101
Vehicle control	5.50	5.51	5.16	5.39	100
No vehicle control	5.69	5.22	5.67	5.53	103
Non-enzymatic iodination control	0.0492*	0.0536*	0.0457*	0.0761	1
No peroxide control	0.0000*	0.0000*	0.0000*	0.0761	1
Plate 2					
Negative control (1 mM DEHP)	5.26	5.69	5.72	5.56	101
Vehicle control	5.68	5.34	5.48	5.50	100
No vehicle control	5.83	5.57	5.66	5.69	103
Non-enzymatic iodination control	0.0344*	0.0403*	0.0413*	0.0761	1
No peroxide control	0.0000*	0.0000*	0.0000*	0.0761	1

The activity of the vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 7
Results obtained for Controls in TPO-Catalyzed Iodination Experiment 5

Sample Type	MIT Concentration (µM)				% Compared to Vehicle Control
	Replicate			Mean	Mean
	A	B	C		
Plate 1					
Negative control (1 mM DEHP)	4.75	4.60	4.22	4.52	92
Vehicle control	5.53	4.60	4.68	4.94	100
No vehicle control	5.36	4.78	4.78	4.98	101
Non-enzymatic iodination control	0.0632*	0.0618*	0.0572*	0.0761	2
No peroxide control	0.0000*	0.0000*	0.0000*	0.0761	2
Plate 2					
Negative control (1 mM DEHP)	5.13	6.08	4.98	5.39	101
Vehicle control	5.43	5.43	5.20	5.35	100
No vehicle control	5.09	5.57	5.05	5.24	98
Non-enzymatic iodination control	0.0270*	0.0584*	0.0681*	0.0761	1
No peroxide control	0.0002*	0.0009*	0.0000*	0.0761	1

The activity of the vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 8
Results obtained for Controls in TPO-Catalyzed Iodination Experiment 6

Sample Type	MIT Concentration (μM)				% Compared to Vehicle Control
	Replicate			Mean	Mean
	A	B	C		
Plate 1					
Negative control (1 mM DEHP)	2.04	2.10	2.13	2.09	100
Vehicle control	2.07	2.10	2.09	2.09	100
No vehicle control	2.26	2.27	2.12	2.22	106
Non-enzymatic iodination control	0.0296*	0.0322*	0.0345*	0.0761	4
No peroxide control	0.0011*	0.0010*	0.0002*	0.0761	4
Plate 2					
Negative control (1 mM DEHP)	2.40	2.34	2.34	2.36	101
Vehicle control	2.42	2.31	2.28	2.34	100
No vehicle control	2.42	2.46	2.38	2.42	104
Non-enzymatic iodination control	0.0361*	0.0386*	0.0395*	0.0761	3
No peroxide control	0.0027*	0.0004*	0.0000*	0.0761	3

The activity of the vehicle control was set at 100%.
* Concentration below LOQ (0.0761 μM). LOQ used for calculation of % compared to vehicle control.

Table 9
Results obtained for the Reference Item PTU in TPO-Catalyzed Iodination Experiment 1

Positive Control Inhibitor	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Plate 1									
PTU 1 nM	5.83	6.67	6.26	6.26	-9.0	97	111	104	104
PTU 10 nM	6.85	6.35	6.40	6.53	-8.0	114	106	106	109
PTU 100 nM	6.36	6.10	6.33	6.26	-7.0	106	101	105	104
PTU 316 nM	5.73	5.92	6.38	6.01	-6.5	95	98	106	100
PTU 1 µM	5.29	5.06	5.08	5.14	-6.0	88	84	84	86
PTU 3.16 µM	1.60	1.57	1.59	1.59	-5.5	27	26	26	26
PTU 10 µM	0.0179*	0.0143*	0.0095*	0.0761	-5.0	1	1	1	1
PTU 31.6 µM	0.0082*	0.0028*	0.0103*	0.0761	-4.5	1	1	1	1
Plate 2									
P2 PTU 31.6 µM	0.0918	0.0401*	0.0577*	0.0813	-4.5	2	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 10
Results obtained for the Reference Item PTU in TPO-Catalyzed Iodination Experiment 2

Positive Control Inhibitor	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Plate 1									
PTU 1 nM	4.67	4.75	4.60	4.67	-9.0	105	107	104	105
PTU 10 nM	4.81	4.98	4.69	4.83	-8.0	108	112	106	109
PTU 100 nM	4.82	4.92	4.78	4.84	-7.0	109	111	108	109
PTU 316 nM	4.46	4.43	4.14	4.34	-6.5	101	100	93	98
PTU 1 µM	3.58	3.53	3.58	3.56	-6.0	81	80	81	80
PTU 3.16 µM	0.481	0.247	0.438	0.388	-5.5	11	6	10	9
PTU 10 µM	0.0042*	0.0063*	0.0066*	0.0761	-5.0	2	2	2	2
PTU 31.6 µM	0.0000*	0.0059*	0.0000*	0.0761	-4.5	2	2	2	2
Plate 2									
P2 PTU 31.6 µM	0.0127*	0.0023*	0.0046*	0.0761	-4.5	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 11
Results obtained for the Reference Item PTU in TPO-Catalyzed Iodination Experiment 3

Positive Control Inhibitor	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Plate 1									
PTU 1 nM	5.77	5.60	5.35	5.57	-9.0	107	104	99	104
PTU 10 nM	5.78	5.89	5.47	5.71	-8.0	107	109	102	106
PTU 100 nM	5.70	5.97	5.61	5.76	-7.0	106	111	104	107
PTU 316 nM	5.33	5.61	5.47	5.47	-6.5	99	104	102	102
PTU 1 µM	6.80 [1)]	4.29	4.43	4.36	-6.0	126 [1)]	80	82	81
PTU 3.16 µM	1.57	1.64	1.58	1.60	-5.5	29	30	29	30
PTU 10 µM	0.0031*	0.0672*	0.0660*	0.0761	-5.0	1	1	1	1
PTU 31.6 µM	0.0000*	0.0000*	0.0030*	0.0761	-4.5	1	1	1	1
Plate 2									
P2 PTU 31.6 µM	0.0029*	0.0000*	0.0034*	0.0761	-4.5	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.
1) Outlier, excluded from calculations.

Table 12
Results obtained for the Reference Item PTU in TPO-Catalyzed Iodination Experiment 5

Positive Control Inhibitor	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Plate 1									
PTU 1 nM	5.21	5.06	5.09	5.12	-9.0	105	103	103	104
PTU 10 nM	5.38	4.88	5.05	5.10	-8.0	109	99	102	103
PTU 100 nM	5.34	4.75	4.97	5.02	-7.0	108	96	101	102
PTU 316 nM	6.37 [1)]	4.65	4.69	4.67	-6.5	129 [1)]	94	95	95
PTU 1 µM	4.25	4.11	4.25	4.20	-6.0	86	83	86	85
PTU 3.16 µM	0.986	0.583	0.556	0.708	-5.5	20	12	11	14
PTU 10 µM	0.0057*	0.0055*	0.0730*	0.0761	-5.0	2	2	2	2
PTU 31.6 µM	0.0040*	0.0000*	0.0000*	0.0761	-4.5	2	2	2	2
Plate 2									
P2 PTU 31.6 µM	0.0017*	0.0000*	0.0017*	0.0761	-4.5	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.
1) Outlier, excluded from calculations.

Table 13
Results obtained for the Reference Item PTU in TPO-Catalyzed Iodination Experiment 6

Positive Control Inhibitor	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Plate 1									
PTU 1 nM	2.38	2.37	2.55	2.44	-9.0	114	114	122	117
PTU 10 nM	2.41	2.45	2.59	2.48	-8.0	115	118	124	119
PTU 100 nM	2.35	2.72	2.29	2.45	-7.0	113	130	110	118
PTU 316 nM	2.20	2.08	2.14	2.14	-6.5	105	100	102	102
PTU 1 µM	1.50	1.59	1.68	1.59	-6.0	72	76	80	76
PTU 3.16 µM	0.0341*	0.0441*	0.0395*	0.0761	-5.5	4	4	4	4
PTU 10 µM	0.0036*	0.0042*	0.0032*	0.0761	-5.0	4	4	4	4
PTU 31.6 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.5	4	4	4	4
Plate 2									
P2 PTU 31.6 µM	0.0002*	0.0072*	0.0000*	0.0761	-4.5	4	4	4	4

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 14
Results obtained for TMTU in TPO-Catalyzed Iodination Experiment 1

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
TMTU 10 nM	6.63	5.94	5.94	6.17	-8.0	110	99	99	103
TMTU 100 nM	6.29	5.91	5.81	6.00	-7.0	105	98	97	100
TMTU 316 nM	6.09	5.67	6.01	5.92	-6.5	101	94	100	98
TMTU 1 µM	4.78	4.92	4.60	4.77	-6.0	79	82	77	79
TMTU 3.16 µM	1.34	0.919	1.38	1.21	-5.5	22	15	23	20
TMTU 10 µM	0.0344*	0.0256*	0.0276*	0.0761	-5.0	1	1	1	1
TMTU 31.6 µM	0.0095*	0.0221*	0.0113*	0.0761	-4.5	1	1	1	1
TMTU 100 µM	0.0022*	0.0036*	0.0026*	0.0761	-4.0	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 15
Results obtained for TMTU in TPO-Catalyzed Iodination Experiment 2

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
TMTU 10 nM	4.64	5.32	4.28	4.75	-8.0	105	120	96	107
TMTU 100 nM	4.32	4.44	4.38	4.38	-7.0	97	100	99	99
TMTU 316 nM	4.11	4.35	4.24	4.23	-6.5	93	98	96	95
TMTU 1 µM	3.29	3.39	3.42	3.37	-6.0	74	76	77	76
TMTU 3.16 µM	0.415	0.436	0.449	0.433	-5.5	9	10	10	10
TMTU 10 µM	0.0199*	0.0132*	0.0208*	0.0761	-5.0	2	2	2	2
TMTU 31.6 µM	0.0148*	0.0013*	0.0000*	0.0761	-4.5	2	2	2	2
TMTU 100 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.0	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 16
Results obtained for TMTU in TPO-Catalyzed Iodination Experiment 3

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
TMTU 10 nM	6.15	5.33	6.13	5.87	-8.0	114	99	114	109
TMTU 100 nM	6.07	5.07	5.76	5.63	-7.0	113	94	107	105
TMTU 316 nM	5.25	5.16	5.28	5.23	-6.5	98	96	98	97
TMTU 1 µM	4.22	4.18	4.44	4.28	-6.0	78	78	82	79
TMTU 3.16 µM	1.01	1.26	1.32	1.20	-5.5	19	23	25	22
TMTU 10 µM	0.0219*	0.0344*	0.0224*	0.0761	-5.0	1	1	1	1
TMTU 31.6 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.5	1	1	1	1
TMTU 100 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.0	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 17
Results obtained for TMTU in TPO-Catalyzed Iodination Experiment 5

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
TMTU 10 nM	5.14	5.36	4.96	5.15	-8.0	104	109	100	104
TMTU 100 nM	5.58	6.06	4.58	5.41	-7.0	113	123	93	110
TMTU 316 nM	4.97	5.21	4.78	4.99	-6.5	101	106	97	101
TMTU 1 µM	4.40	3.96	3.62	3.99	-6.0	89	80	73	81
TMTU 3.16 µM	0.692	0.741	1.13	0.853	-5.5	14	15	23	17
TMTU 10 µM	0.0273*	0.0260*	0.0314*	0.0761	-5.0	2	2	2	2
TMTU 31.6 µM	0.0005*	0.0006*	0.0012*	0.0761	-4.5	2	2	2	2
TMTU 100 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.0	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 18
Results obtained for TMTU in TPO-Catalyzed Iodination Experiment 6

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
TMTU 10 nM	2.51	2.29	2.29	2.36	-8.0	120	110	110	113
TMTU 100 nM	2.33	2.30	2.54	2.39	-7.0	112	110	122	115
TMTU 316 nM	2.36	1.94	2.14	2.15	-6.5	113	93	103	103
TMTU 1 µM	1.43	1.48	1.34	1.42	-6.0	69	71	64	68
TMTU 3.16 µM	0.155	0.117	0.109	0.127	-5.5	7	6	5	6
TMTU 10 µM	0.0084*	0.0097*	0.0131*	0.0761	-5.0	4	4	4	4
TMTU 31.6 µM	0.0000*	0.0001*	0.0006*	0.0761	-4.5	4	4	4	4
TMTU 100 µM	0.0000*	0.0000*	0.0000*	0.0761	-4.0	4	4	4	4

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 19
Results obtained for Methimazole in TPO-Catalyzed Iodination Experiment 1

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Methimazole 10 nM	5.83	5.78	5.92	5.84	-8.0	97	96	98	97
Methimazole 100 nM	5.48	5.33	5.51	5.44	-7.0	91	89	92	90
Methimazole 316 nM	4.30	4.47	4.80	4.52	-6.5	71	74	80	75
Methimazole 1 µM	0.0170*	0.0208*	0.0173*	0.0761	-6.0	1	1	1	1
Methimazole 3.16 µM	0.0016*	0.0028*	0.0082*	0.0761	-5.5	1	1	1	1
Methimazole 10 µM	0.0099*	0.0000*	0.0004*	0.0761	-5.0	1	1	1	1
Methimazole 31.6 µM	0.0274*	0.0057*	0.0000*	0.0761	-4.5	1	1	1	1
Methimazole 100 µM	0.0124*	0.0000*	0.0014*	0.0761	-4.0	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 20
Results obtained for Methimazole in TPO-Catalyzed Iodination Experiment 2

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Methimazole 3.16 nM	4.97	4.72	4.57	4.75	-8.5	112	106	103	107
Methimazole 10 nM	4.72	4.48	4.62	4.61	-8.0	106	101	104	104
Methimazole 31.6 nM	4.48	4.39	4.39	4.42	-7.5	101	99	99	100
Methimazole 100 nM	4.09	4.43	4.30	4.27	-7.0	92	100	97	96
Methimazole 316 nM	2.96	2.86	2.80	2.87	-6.5	67	64	63	65
Methimazole 1 µM	0.0112*	0.0079*	0.0142*	0.0761	-6.0	2	2	2	2
Methimazole 3.16 µM	0.0009*	0.0132*	0.0145*	0.0761	-5.5	2	2	2	2
Methimazole 10 µM	0.0000*	0.0000*	0.0183*	0.0761	-5.0	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 21
Results obtained for Methimazole in TPO-Catalyzed Iodination Experiment 3

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Methimazole 3.16 nM	5.53	5.34	5.46	5.44	-8.5	103	99	101	101
Methimazole 10 nM	5.76	6.89	5.48	6.05	-8.0	107	128	102	112
Methimazole 31.6 nM	5.05	5.47	5.73	5.42	-7.5	94	102	106	101
Methimazole 100 nM	5.21	5.23	5.18	5.21	-7.0	97	97	96	97
Methimazole 316 nM	10.1 [1)]	4.09	4.09	4.09	-6.5	188 [1)]	76	76	76
Methimazole 1 µM	0.0205*	0.0144*	0.0178*	0.0761	-6.0	1	1	1	1
Methimazole 3.16 µM	0.0000*	0.0302*	0.0008*	0.0761	-5.5	1	1	1	1
Methimazole 10 µM	0.0000*	0.0024*	0.0182*	0.0761	-5.0	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.
[1)] Outlier, excluded from calculations.

Table 22
Results obtained for Methimazole in TPO-Catalyzed Iodination Experiment 5

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Methimazole 3.16 nM	5.00	4.84	4.64	4.83	-8.5	101	98	94	98
Methimazole 10 nM	7.73 [1)]	5.43	4.99	5.21	-8.0	157 [1)]	110	101	106
Methimazole 31.6 nM	3.56 [2)]	3.46 [2)]	3.40 [2)]	NA	-7.5	72 [1)]	70 [1)]	69 [1)]	NA
Methimazole 100 nM	5.03	4.67	4.42	4.71	-7.0	102	95	90	95
Methimazole 316 nM	0.0001*	0.0015*	0.0006*	0.0761	-6.5	2	2	2	2
Methimazole 1 µM	0.0170*	0.0117*	0.0137*	0.0761	-6.0	2	2	2	2
Methimazole 3.16 µM	0.0036*	0.0000*	0.0012*	0.0761	-5.5	2	2	2	2
Methimazole 10 µM	0.0000*	0.0000*	0.0000*	0.0761	-5.0	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicates.
[2)] Outlier, complete concentration excluded from calculations as results differed significantly from the results obtained for the other experiments with methimazole.

Table 23
Results obtained for Methimazole in TPO-Catalyzed Iodination Experiment 6

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Methimazole 3.16 nM	2.51	2.31	2.44	2.42	-8.5	120	111	117	116
Methimazole 10 nM	2.72	2.11	2.56	2.46	-8.0	130	101	123	118
Methimazole 31.6 nM	2.31	2.15	2.29	2.25	-7.5	111	103	110	108
Methimazole 100 nM	2.09	2.00	2.06	2.05	-7.0	100	96	99	98
Methimazole 316 nM	1.72	1.37	1.23	1.44	-6.5	83	66	59	69
Methimazole 1 µM	0.0092*	0.0083*	0.0065*	0.0761	-6.0	4	4	4	4
Methimazole 3.16 µM	0.0003*	0.0009*	0.0008*	0.0761	-5.5	4	4	4	4
Methimazole 10 µM	0.0000*	0.0000*	0.0000*	0.0761	-5.0	4	4	4	4

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 24
Results obtained for Naringenin in TPO-Catalyzed Iodination Experiment 1

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Naringenin 10 nM	7.65	6.60	5.74	6.66	-8.0	130	112	98	113
Naringenin 100 nM	5.54	5.11	5.13	5.26	-7.0	94	87	87	89
Naringenin 316 nM	3.65	4.09	4.24	3.99	-6.5	62	70	72	68
Naringenin 1 µM	1.64	1.32	1.21	1.39	-6.0	28	22	21	24
Naringenin 3.16 µM	0.0999	0.137	0.141	0.126	-5.5	2	2	2	2
Naringenin 10 µM	0.0524*	0.0482*	0.0721*	0.0761	-5.0	1	1	1	1
Naringenin 31.6 µM	0.0296*	0.0210*	0.0426*	0.0761	-4.5	1	1	1	1
Naringenin 100 µM	0.0210*	0.0181*	0.0714*	0.0761	-4.0	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 25
Results obtained for Naringenin in TPO-Catalyzed Iodination Experiment 2

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Naringenin 10 nM	4.15	4.31	4.09	4.18	-8.0	104	108	103	105
Naringenin 31.6 nM	5.04	4.23	4.10	4.46	-7.5	127	106	103	112
Naringenin 100 nM	3.61	3.49	3.41	3.50	-7.0	91	88	86	88
Naringenin 316 nM	2.51	2.79	2.65	2.65	-6.5	63	70	67	67
Naringenin 1 µM	0.772	0.842	1.21	0.943	-6.0	19	21	31	24
Naringenin 3.16 µM	0.0765	0.0672*	0.0567*	0.0762	-5.5	2	2	2	2
Naringenin 10 µM	0.0095*	0.0098*	0.0058*	0.0761	-5.0	2	2	2	2
Naringenin 31.6 µM	0.0050*	0.0032*	0.0157*	0.0761	-4.5	2	2	2	2

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 26
Results obtained for Naringenin in TPO-Catalyzed Iodination Experiment 3

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Naringenin 10 nM	5.68	5.80	5.72	5.73	-8.0	103	105	104	104
Naringenin 31.6 nM	6.10	5.62	5.54	5.75	-7.5	111	102	101	105
Naringenin 100 nM	4.81	4.95	5.01	4.92	-7.0	87	90	91	90
Naringenin 316 nM	4.45	3.78	3.68	3.97	-6.5	81	69	67	72
Naringenin 1 µM	1.67	1.56	1.55	1.59	-6.0	30	28	28	29
Naringenin 3.16 µM	0.0796	0.0738*	0.0782	0.0780	-5.5	1	1	1	1
Naringenin 10 µM	0.0097*	0.0403*	0.0101*	0.0761	-5.0	1	1	1	1
Naringenin 31.6 µM	0.0003*	0.0000*	0.0009*	0.0761	-4.5	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 27
Results obtained for Naringenin in TPO-Catalyzed Iodination Experiment 5

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Naringenin 10 nM	5.08	5.53	5.36	5.32	-8.0	95	103	100	99
Naringenin 31.6 nM	5.36	5.02	4.89	5.09	-7.5	100	94	91	95
Naringenin 100 nM	5.10	4.36	4.73	4.73	-7.0	95	81	88	88
Naringenin 316 nM	4.12	3.57	3.63	3.77	-6.5	77	67	68	70
Naringenin 1 µM	1.53	1.33	1.26	1.37	-6.0	29	25	24	26
Naringenin 3.16 µM	0.0856	0.0652*	0.0825	0.0814	-5.5	2	1	2	2
Naringenin 10 µM	0.0165*	0.0125*	0.0103*	0.0761	-5.0	1	1	1	1
Naringenin 31.6 µM	0.0003*	0.0000*	0.0015*	0.0761	-4.5	1	1	1	1

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 28
Results obtained for Naringenin in TPO-Catalyzed Iodination Experiment 6

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Naringenin 10 nM	2.63	2.53	2.58	2.58	-8.0	113	108	110	110
Naringenin 31.6 nM	2.63	2.30	2.41	2.45	-7.5	113	98	103	105
Naringenin 100 nM	2.11	2.05	1.99	2.05	-7.0	90	88	85	88
Naringenin 316 nM	1.43	1.35	1.34	1.37	-6.5	61	58	57	59
Naringenin 1 µM	0.307	0.297	0.318	0.307	-6.0	13	13	14	13
Naringenin 3.16 µM	0.0398*	0.0340*	0.0375*	0.0761	-5.5	3	3	3	3
Naringenin 10 µM	0.0099*	0.0073*	0.0070*	0.0761	-5.0	3	3	3	3
Naringenin 31.6 µM	0.0055*	0.0006*	0.0000*	0.0761	-4.5	3	3	3	3

The activity of the corresponding vehicle control was set at 100%.
* Concentration below LOQ (0.0761 µM). LOQ used for calculation of % compared to vehicle control.

Table 29
Results obtained for Flavanone in TPO-Catalyzed Iodination Experiment 1

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Flavanone 10 nM	5.54	6.08	7.06 [1)]	5.81	-8.0	94	103	120 [1)]	99
Flavanone 100 nM	5.38	5.42	6.14 [1)]	5.40	-7.0	92	92	104 [1)]	92
Flavanone 316 nM	5.52	5.34	5.39 [1)]	5.43	-6.5	94	91	92 [1)]	92
Flavanone 1 µM	5.81	5.58	5.60 [1)]	5.70	-6.0	99	95	95 [1)]	97
Flavanone 3.16 µM	5.61	5.42	4.55 [1)]	5.51	-5.5	95	92	77 [1)]	94
Flavanone 10 µM	5.57	5.51	3.01 [1)]	5.54	-5.0	95	94	51 [1)]	94
Flavanone 31.6 µM	5.82	5.53	1.23 [1)]	5.67	-4.5	99	94	21 [1)]	97
Flavanone 100 µM	5.81	4.97	0.189 [1)]	5.39	-4.0	99	85	3 [1)]	92

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicate series.

Table 30
Results obtained for Flavanone in TPO-Catalyzed Iodination Experiment 2

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Flavanone 10 nM	3.96	4.86	4.63	4.48	-8.0	100	122	116	113
Flavanone 100 nM	4.09	4.08	4.26	4.15	-7.0	103	103	107	104
Flavanone 316 nM	3.94	4.04	4.11	4.03	-6.5	99	102	103	101
Flavanone 1 µM	3.96	4.22	3.97	4.05	-6.0	100	106	100	102
Flavanone 3.16 µM	4.23	3.75	3.79	3.92	-5.5	106	94	95	99
Flavanone 10 µM	3.91	3.81	4.11	3.94	-5.0	98	96	104	99
Flavanone 31.6 µM	4.32	4.27	4.29	4.30	-4.5	109	108	108	108
Flavanone 100 µM	4.44	5.42	3.95	4.60	-4.0	112	136	100	116

The activity of the corresponding vehicle control was set at 100%.

Table 31
Results obtained for Flavanone in TPO-Catalyzed Iodination Experiment 3

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Flavanone 10 nM	5.59	5.64	5.86	5.70	-8.0	102	103	107	104
Flavanone 100 nM	5.44	5.65	5.68	5.59	-7.0	99	103	103	102
Flavanone 316 nM	5.98	5.74	5.71	5.81	-6.5	109	104	104	106
Flavanone 1 µM	5.70	5.68	6.10	5.83	-6.0	104	103	111	106
Flavanone 3.16 µM	6.18	6.30	6.02	6.17	-5.5	112	115	109	112
Flavanone 10 µM	5.91	5.36	5.82	5.70	-5.0	107	97	106	104
Flavanone 31.6 µM	5.76	5.41	5.77	5.65	-4.5	105	98	105	103
Flavanone 100 µM	5.90	5.60	5.13	5.55	-4.0	107	102	93	101

The activity of the corresponding vehicle control was set at 100%.

Table 32
Results obtained for Flavanone in TPO-Catalyzed Iodination Experiment 5

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Flavanone 10 nM	5.69	5.07	5.49	5.42	-8.0	106	95	103	101
Flavanone 100 nM	5.40	4.75	5.15	5.10	-7.0	101	89	96	95
Flavanone 316 nM	5.47	5.04	5.13	5.21	-6.5	102	94	96	97
Flavanone 1 µM	5.12	4.94	5.00	5.02	-6.0	96	92	93	94
Flavanone 3.16 µM	6.03	5.61	5.12	5.59	-5.5	113	105	96	104
Flavanone 10 µM	5.21	4.88	4.94	5.01	-5.0	97	91	92	94
Flavanone 31.6 µM	5.42	4.91	4.99	5.11	-4.5	101	92	93	95
Flavanone 100 µM	5.43	4.86	4.72	5.00	-4.0	102	91	88	94

The activity of the corresponding vehicle control was set at 100%.

Table 33
Results obtained for Flavanone in TPO-Catalyzed Iodination Experiment 6

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Flavanone 10 nM	2.51	2.82	2.50	2.61	-8.0	108	121	107	112
Flavanone 100 nM	2.42	2.46	2.49	2.45	-7.0	104	105	106	105
Flavanone 316 nM	2.47	2.47	2.44	2.46	-6.5	106	106	105	105
Flavanone 1 µM	2.42	2.44	2.51	2.45	-6.0	103	104	107	105
Flavanone 3.16 µM	2.50	2.36	2.40	2.42	-5.5	107	101	103	104
Flavanone 10 µM	2.45	2.41	2.37	2.41	-5.0	105	103	101	103
Flavanone 31.6 µM	2.32	2.49	2.35	2.39	-4.5	99	107	101	102
Flavanone 100 µM	3.20 [1)]	2.53	2.34	2.44	-4.0	137 [1)]	108	100	104

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicates.

Table 34
Results obtained for Sulfamethazine in TPO-Catalyzed Iodination Experiment 1

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Sulfamethazine 10 nM	7.10	8.79 [1)]	6.13	6.62	-8.0	121	149	104	113
Sulfamethazine 100 nM	6.21	5.65	5.58	5.81	-7.0	106	96	95	99
Sulfamethazine 316 nM	5.57	6.11	5.70	5.79	-6.5	95	104	97	99
Sulfamethazine 1 µM	5.29	5.27	5.20	5.25	-6.0	90	90	89	89
Sulfamethazine 3.16 µM	4.49	4.45	4.44	4.46	-5.5	76	76	76	76
Sulfamethazine 10 µM	2.99	3.02	3.07	3.03	-5.0	51	51	52	52
Sulfamethazine 31.6 µM	1.20	1.43	1.16	1.27	-4.5	20	24	20	22
Sulfamethazine 100 µM	0.191	0.188	0.226	0.202	-4.0	3	3	4	3

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicates and the next concentration.

Table 35
Results obtained for Sulfamethazine in TPO-Catalyzed Iodination Experiment 2

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Sulfamethazine 10 nM	5.15 [1)]	4.08	3.95	4.02	-8.0	130 [1)]	103	100	101
Sulfamethazine 100 nM	4.05	4.65	4.11	4.27	-7.0	102	117	103	108
Sulfamethazine 316 nM	4.01	4.28	4.00	4.09	-6.5	101	108	101	103
Sulfamethazine 1 µM	4.11	3.42	3.71	3.75	-6.0	104	86	93	94
Sulfamethazine 3.16 µM	3.05	3.17	3.47	3.23	-5.5	77	80	87	81
Sulfamethazine 10 µM	2.07	2.26	2.22	2.18	-5.0	52	57	56	55
Sulfamethazine 31.6 µM	0.866	0.948	0.788	0.867	-4.5	22	24	20	22
Sulfamethazine 100 µM	0.222	0.174	0.157	0.184	-4.0	6	4	4	5

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicates.

Table 36
Results obtained for Sulfamethazine in TPO-Catalyzed Iodination Experiment 3

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Sulfamethazine 10 nM	6.12	5.74	6.23	6.03	-8.0	111	104	113	110
Sulfamethazine 100 nM	6.05	5.59	5.61	5.75	-7.0	110	102	102	104
Sulfamethazine 316 nM	5.88	7.00	5.48	6.12	-6.5	107	127	100	111
Sulfamethazine 1 µM	5.21	6.75	5.37	5.78	-6.0	95	123	98	105
Sulfamethazine 3.16 µM	4.68	4.41	4.38	4.49	-5.5	85	80	80	82
Sulfamethazine 10 µM	3.47	3.02	3.26	3.25	-5.0	63	55	59	59
Sulfamethazine 31.6 µM	1.44	1.16	1.31	1.30	-4.5	26	21	24	24
Sulfamethazine 100 µM	0.260	0.258	0.220	0.246	-4.0	5	5	4	4

The activity of the corresponding vehicle control was set at 100%.

Table 37
Results obtained for Sulfamethazine in TPO-Catalyzed Iodination Experiment 5

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Sulfamethazine 10 nM	3.46 [1)]	5.39	5.16	5.27	-8.0	65 [1)]	101	96	99
Sulfamethazine 100 nM	5.25	5.03	5.54	5.28	-7.0	98	94	104	99
Sulfamethazine 316 nM	5.34	4.79	5.07	5.07	-6.5	100	90	95	95
Sulfamethazine 1 µM	4.67	5.31	4.84	4.94	-6.0	87	99	90	92
Sulfamethazine 3.16 µM	4.86	4.33	4.16	4.45	-5.5	91	81	78	83
Sulfamethazine 10 µM	2.84	2.49	2.82	2.71	-5.0	53	46	53	51
Sulfamethazine 31.6 µM	1.04	1.02	0.881	0.980	-4.5	19	19	16	18
Sulfamethazine 100 µM	0.178	0.163	0.185	0.175	-4.0	3	3	3	3

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicates.

Table 38
Results obtained for Sulfamethazine in TPO-Catalyzed Iodination Experiment 6

Test Item	MIT Concentration (µM)				Log Conc. (M)	% Activity of Vehicle Control			
	Replicate			Mean		Replicate			Mean
	#1	#2	#3			#1	#2	#3	
Sulfamethazine 10 nM	3.11	6.35 [1)]	2.62	2.87	-8.0	133	272 [1)]	112	123
Sulfamethazine 100 nM	2.81	6.45 [1)]	2.44	2.63	-7.0	120	276 [1)]	105	113
Sulfamethazine 316 nM	2.71	6.17 [1)]	2.49	2.60	-6.5	116	264 [1)]	106	111
Sulfamethazine 1 µM	2.29	5.98 [1)]	2.22	2.25	-6.0	98	256 [1)]	95	97
Sulfamethazine 3.16 µM	1.84	5.40 [1)]	1.85	1.85	-5.5	79	231 [1)]	79	79
Sulfamethazine 10 µM	1.17	4.05 [1)]	1.20	1.19	-5.0	50	174 [1)]	51	51
Sulfamethazine 31.6 µM	0.387	2.14 [1)]	0.383	0.385	-4.5	17	92 [1)]	16	16
Sulfamethazine 100 µM	0.123	0.558 [1)]	0.0792	0.101	-4.0	5	24 [1)]	3	4

The activity of the corresponding vehicle control was set at 100%.
[1)] Outlier, excluded from calculations as results differed significantly from the other two replicate series.

Appendix 1
UPLC-MS/MS Conditions for the Analysis of MIT

Table 39
UPLC-MS/MS Conditions for the Analysis of MIT in Incubation Samples

Mobile Phase:	A: 0.1% FA in MQ B: 0.1% FA in ACN	
	Gradient: 0.0-0.2 min 0.2-1.2 min 1.2-1.5 min 1.5-1.9 min 1.9-2.0 min 2.0-2.5 min	95% A - 5% B 50% A - 50% B 5% A - 95% B 5% A - 95% B 95% A - 5% B 95% A - 5% B
Flow Rate :	0.4 mL/min	
Column:	Acquity UPLC BEH C18 50 mm × 2.1 mm ID, 1.7 µm particle size	
Column oven temperature:	Set at 40ºC	
Sample tray temperature:	Set at 8ºC	
Injection volume:	3 µL	
Detection:	Ionisation source: Capillary voltage: Source temperature: Source parameters:	ESI+ 1.0 kV 120ºC x = 6 z = 6
	Collision Gas Flow Desolvation Temp. Cone Gas Flow Desolvation Gas Flow	0.18 mL/min 500ºC 150 L/Hr 1000 L/Hr
	MRM monitored MIT Dwell Time	 *m/z* 307.75 > *m/z* 134.9 Collision energy: 26 eV Cone voltage: 20 V Measured from 0.6 – 2.50 min 100 ms.
	MIT-$^{13}C_6$ Dwell Time	*m/z* 313.75 > *m/z* 140.9 Collision energy: 26 eV Cone voltage: 20 V Measured from 0.6 – 2.50 min 50 ms.
	Divert Valve:	0.0 min to waste / 0.5 min to MS / 1.5 min to waste.

Appendix 2
Deviation and Study Plan

DEVIATION

All deviations that occurred during the study have been authorized/acknowledged by the Study Director, assessed for impact, and documented in the study records. All study plan deviations and those SOP deviations that could have impacted the quality or integrity of the study are listed below.

None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

Preparation of MIT Calibration Standards and QC Samples; Buffer pH

- The pH of the 0.1 M potassium phosphate buffer that was used for the preparation of the MIT calibration standards and QC samples on the 05th of October 2021 was 7.8 instead of 7.4 as mentioned in the Study Plan. These MIT calibration standards and QC samples were used for sample analysis on the 05th, the 06th and the 07th of October 2021.

  Evaluation: Acceptance criteria for the calibration curves and QC samples analyzed were all met and were in line with the results obtained for calibration standards and QC samples prepared correctly. This demonstrates that the incorrect buffer pH did not affect the integrity of the calibration standards and QC samples or the interpretation of the study results and conclusions.

FINAL STUDY PLAN

Test Facility Study No. 20309164

In Vitro Suppression of Thyroid Peroxidase (TPO)-Catalyzed Iodination using FTC-238-hrTPO Cell Homogenates

GLP

SPONSOR:
European Commission (DG-JRC)
Directorate F - Health, Consumers and Reference Materials
Unit F3 - Chemical Safety and Alternative Methods / The European Union Reference Laboratory for Alternatives to Animal Testing
(EURL ECVAM)
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1. OBJECTIVE

This study is performed for PART 1 of the EURL ECVAM coordinated Thyroid Validation Study. After the full description of method 2C (Tyrosine iodination using liquid chromatography) in standard operating procedures (SOPs), in this study the robustness and reliability of the method to determine the suppression of human thyroid peroxidase (TPO)-catalyzed iodination will be assessed *in vitro*. This will be done by performing five valid runs with the reference item 6-propyl-2-thiouracil (PTU, CAS# 51-52-5) and the test items flavanone, methimazole, N,N,N,N-tetramethylthiourea (TMTU), naringenin and sulfamethazine.

TPO is an enzyme which is present on the apical membrane of thyroid follicular cells where it reduces hydrogen peroxide (H_2O_2), thereby elevating the oxidation state of iodide to an iodinating species (often considered to be hypoiodous acid), and iodinates tyrosyl residues in the thyroglobulin (Tg) glycoprotein. Initial iodination of Tg produces monoiodotyrosine (MIT) and diiodotyrosine (DIT) while subsequent oxidation of MIT and DIT by TPO to radical species couples two residues of DIT, both still linked to the Tg, to produce thyroxine (T4) and couples one residue of MIT and one residue of DIT to produce triiodothyronine (T3). When thyroid hormones are needed, hormone-rich Tg is taken up into thyroid epithelial cells by endocytosis and digested by proteases which results in the release of T4 and T3 into the blood circulation through the action of their transporters. Chemicals potentially can suppress TPO-catalyzed iodination and/or coupling and in that way alter thyroid hormone homeostasis *in vivo*.

FTC-238-hrTPO cells are human thyroid carcinoma cells stably transfected with an expression clone coding for human recombinant (hr) TPO and can be used to prepare cell lysates containing the hrTPO enzyme. To evaluate potential interference with TPO-catalyzed iodination, FTC-238-hrTPO cell lysates can be incubated with L-tyrosine, potassium iodide and H_2O_2 in the presence or absence of a test item. During incubation, TPO enzymatically converts L-tyrosine into MIT and formation of this metabolite can be monitored by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as a direct measurement of TPO-catalyzed iodination. A small amount of MIT may also be formed non-enzymatically, its formation will be assessed in separate incubations.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual dates will be included in the Final Report.

Experimental Start Date:	06 Sep 2021 (First date of study-specific data collection)
Experimental Completion Date:	14 Nov 2021 (Last date data are collected from the study)
Unaudited Draft Report:	28 Nov 2021

3. SPONSOR

Role	Name	Contact Information
Sponsor Representative/ Study Monitor	Ingrid Langezaal	Address as cited for Sponsor E-mail: EU-Thyroid@ec.europa.eu

4. RESPONSIBLE PERSONNEL

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Jelle Reinen, PhD, ERT	Address as cited for Test Facility
Test Facility Management	Charles River	Beppy van de Waart, MSc, ERT	Address as cited for Test Facility
Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility

5. TEST MATERIALS

5.1. Test Item Characterization

Documentation of the identity, strength, purity, composition, and stability for the test items is available at the Test Facility. A Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report.

5.2. Test Material Identification

5.2.1. Test Items

5.2.1.1. Flavanone

Identification: Flavanone

Batch (Lot) Number: MKCG6841

Expiry date: 19 June 2023

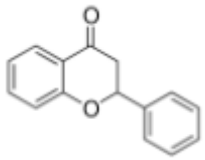
Physical Description: White powder

Purity/Composition: See Certificate of Analysis issued 19 June 2018

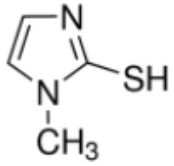
Storage Conditions: At room temperature

Additional information

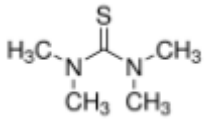
Test Facility test item number: 212559/A

Purity/Composition correction factor:	No correction factor required
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	2,3-Dihydroflavone (Flavanone)
CAS number:	487-26-3
Molecular structure:	
Molecular formula:	$C_{15}H_{12}O_2$
Molecular weight:	224.25 g/mol

5.2.1.2. Methimazole

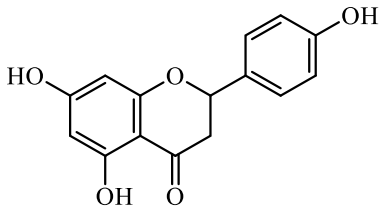
Identification:	Methimazole
Batch (Lot) Number:	WXBC8588V
Expiry date:	19 October 2023
Physical Description:	Off-white powder
Purity/Composition:	See Certificate of Analysis issued 19 October 2018
Storage Conditions:	At room temperature
Additional information	
Test Facility test item number:	212556/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	2-Mercapro-1-methylimidazole (MMI) 1-Methyl-2-imidazolethiol
CAS number:	60-56-0
Molecular structure:	
Molecular formula:	$C_4H_6N_2S$
Molecular weight:	114.17 g/mol

5.2.1.3. N,N,N,N-tetramethylthiourea

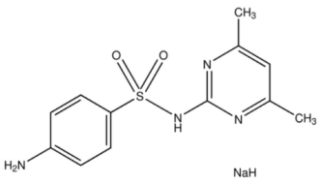
Identification:	N,N,N,N-tetramethylthiourea
Batch (Lot) Number:	SHBJ4707
Expiry date:	02 October 2022
Physical Description:	White powder
Purity/Composition:	See Certificate of Analysis issued 02 October 2017
Storage Conditions:	In refrigerator (2-8°C)
Additional information	
Test Facility test item number:	212555/A
Purity/Composition correction factor:	No correction factor required
Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	1,1,3,3-tetramethyl-2-thiourea
CAS number:	2782-91-4
Molecular structure:	
Molecular formula:	$C_5H_{12}N_2S$
Molecular weight:	132.23 g/mol

5.2.1.4. Naringenin

Identification:	Naringenin
Batch (Lot) Number:	A63107
Expiry date:	03 January 2024
Physical Description:	White solid
Purity/Composition:	See Certificate of Analysis issued 03 January 2019
Storage Conditions:	At room temperature
Additional information	
Test Facility test item number:	212557/A
Purity/Composition correction factor:	No correction factor required

Test item handling:	No specific handling conditions required
Chemical name (IUPAC, synonym or trade name):	(2R)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one (4',5,7-Trihydroxyflavanone)
CAS number:	480-41-1
Molecular structure:	
Molecular formula:	$C_{15}H_{12}O_5$
Molecular weight:	272.3 g/mol

5.2.1.5. Sulfamethazine Sodium Salt

Identification:	Sulfamethazine sodium salt
Batch (Lot) Number:	WXBD0389V
Expiry date:	30 June 2023 (retest date)
Physical Description:	White powder
Purity/Composition:	100%
Storage Conditions:	In refrigerator (2-8°C)
Additional information	
Test Facility test item number:	RS739
Purity/Composition correction factor:	No correction factor required
CAS number:	1981-58-4
Molecular structure:	
Molecular formula:	$C_{12}H_{13}N_4NaO_2S$
Molecular weight:	300.31 g/mol

5.2.2. Control Items

5.2.2.1. Vehicle

Dimethyl sulfoxide (DMSO) or another appropriate solvent will be used as vehicle.

5.2.2.2. Substrate

L-tyrosine (CAS# 60-18-4) will be used as substrate for the TPO-catalyzed iodination assay. Details on the supplier and batch will be included in the Final Report.

5.2.2.3. Metabolite

Monoiodotyrosine (MIT) (CAS# 70-78-0, AS2084) is the metabolite that will be evaluated in the TPO-catalyzed iodination assay. Details on the supplier and batch will be included in the Final Report.

5.2.2.4. Internal Standard

Monoiodotyrosine-$^{13}C_6$ (MIT-$^{13}C_6$) (AS2083) will be used as internal standard (IS) in the TPO-catalyzed iodination assay. Details on the supplier and batch will be included in the Final Report.

5.2.2.5. Negative Control

Bis-(2-ethylhexyl)-phthalate (DEHP, CAS# 117-81-7, RS493) will be used as a negative control. Details on the supplier will be included in the Final Report.

5.2.2.6. Reference Item

6-Propyl-2-thiouracil (PTU, CAS# 51-52-5, RS506) will be used as a reference item. Details on the supplier and batch will be included in the Final Report.

5.3. Reserve Samples

For each batch (lot) of test and control item, if practically possible, a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility.

5.4. Test and Control Item and Internal Standard Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test materials will be maintained.

5.5. Safety

The following safety instruction(s) apply to this study:

- Standard safety precautions specified in Charles River Den Bosch procedures
- Specific safety precautions are provided in the Charles River Den Bosch internal EH&S test item risk assessment

6. DOSE FORMULATION AND ANALYSIS

Test item amounts may be pre-weighed for a part of- or even the entire study at the discretion of the Study Director when stored under the same conditions as prescribed for the bulk container.

6.1. Preparation of Test Item

No correction for the purity/composition of the test items will be performed.

Before performing the first TPO-catalyzed iodination experiment, a solubility test will be performed for each test item to determine the appropriate concentration ranges to be tested

(see Section 8.2.1). Each test item will be tested at eight concentrations in the TPO-catalyzed iodination assay.

6.1.1. Stock Solutions of the Test Items

Stock solutions of the test items will be prepared in DMSO or another appropriate solvent on the day of use.

For each test item, the selection of the solvent will be approved by the Study Director in the study files. Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.1.2. Spiking Solutions of the Test Items

For each test item, the stock solution will be further diluted in DMSO or another appropriate solvent in a log or half-log fashion to obtain eight 100× spiking solutions. The spiking solutions will be further diluted in the incubation mixtures. The final exposure concentrations in the TPO-catalyzed iodination assay will be e.g., 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM and 1 mM. These target concentrations may change if solubility, or other limitations, are encountered and may vary between experiments. The actual final test item concentrations used in the TPO-catalyzed iodination assay experiments will be included in the Final Report. The final concentration of the solvent in the incubation mixtures will be the same for all incubations and will not exceed 1% (v/v).

Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.1.3. Preparation of Control Item Solutions

6.1.3.1. Preparation of DEHP Solutions

A 100 mM stock solution will be prepared in DMSO or another appropriate solvent and will be stored in the freezer set to maintain -20°C for a maximum of one month. The 100 mM stock solution will be further diluted in the incubation mixture. The final concentration in the incubation mixtures will be 1 mM.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.1.3.2. Preparation of PTU Solutions

A 10 mM stock solution will be prepared in DMSO or another appropriate solvent on the day of use. The stock solution will be diluted to achieve eight 100× concentrations in solvent. The resulting spike solutions will be further diluted in the incubation mixture. The final concentrations in the incubation mixture will be 10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM, 31.6 µM and 100 µM.

PTU spiking solutions will be prepared freshly on the day of use. Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.1.4. Preparation of Internal Standard Solutions

No correction will be made for the purity/composition of the internal standard (IS).

A stock solution of the IS will be prepared in 0.1 M HCl at a target concentration of 1 mg/mL. The stock solution will be aliquoted in glass vials and stored in the freezer set to maintain -20°C.

The IS stock solution will be diluted in methanol containing 300 µM sodium thiosulfate to obtain a solution with a final IS concentration of 3000 ng/mL (IS working solution). If applicable, additional IS working solutions with a different final IS concentration may be prepared.

IS working solutions will be prepared freshly on the day of use. Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.2. Sample Collection and Analysis

Analysis of test item in vehicle for concentration, stability, homogeneity will not be performed, however, to limit the impact, the test item preparation will be performed with approved procedures and documented in detail. Formulations will be visually inspected for homogeneity prior to use and all formulations will be used within 4 hours after adding vehicle to the test item. This GLP exception will therefore be considered as being minor with no impact on the outcomes and the integrity and the achievement of the objective of the study.

7. TEST SYSTEM

Test system	FTC-238-hrTPO cell lysates.
Rationale	The FTC-238 human follicular thyroid carcinoma cell line was established from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old male. The cells are polymorphic showing flat polygonal to spindle-like morphologies. The FTC-238 cells are genetically modified to incorporate human recombinant TPO and a neomycin resistance gene. Prepared cell lysates of hematin-stimulated FTC-238-hrTPO cells contain active human thyoid peroxidase.
Source	The FTC-238-hrTPO cell line was provided by the study Sponsor EURL ECVAM, who obtained it from Charité.
Storage	FTC-238-hrTPO cell lysates are stored in an ultra-low freezer set to maintain -80°C.

8. EXPERIMENTAL DESIGN

8.1. Study Design

Incubations will be performed with FTC-238-hrTPO cell lysates and the test items to evaluate the possible suppressive effect of each test item on the TPO-catalyzed iodination by measuring the formation of MIT.

Five independent and valid runs will be performed for each test item. In case of invalid results, a maximum of eight runs will be performed. In case two consecutive invalid runs occur, the Study Director will inform the Sponsor EURL ECVAM.

During the first (range finding) experiment, it is recommended to use the highest possible test item concentration, however, the highest (nominal) concentration to be tested should be at least 10^{-5} M irrespective of the test item solubility. When concentrations equal or lower than 10^{-5} M are insoluble, this will be noted in the raw data files of the study and mentioned in the

Final Report. The lowest concentration to be tested is generally 10^{-10} M. If the highest concentration to be tested is lower than $10^{-3.5}$ M, mid-log concentration(s) will be added near the lower end of the curve to obtain a total of eight test item concentrations. If during the first experiment the top of the curve (control iodination activity) is not reached at the lowest test item concentration, lower concentrations will be selected for the second experiment and, only if applicable, subsequent experiment(s).

8.2. Main Study

8.2.1. Solubility Test

For each test item, a preliminary test will be performed to determine whether the test item may have any solubility problems, i.e., the presence of cloudiness or precipitate will be evaluated. The test item will be dissolved in an appropriate vehicle (e.g., DMSO) to prepare a stock solution at an initial concentration of 100 mM. The presence of cloudiness or precipitate will be evaluated by visual inspection and under the microscope. If necessary, the test item stock solution will be further diluted (e.g., ½ log lower) to define the highest soluble concentration of test item in vehicle.

If the test item is soluble in vehicle, a 100-fold dilution of the stock solution will be prepared in the incubation buffer and it will be determined whether the test item may have any solubility problems in the incubation buffer, i.e., the presence of cloudiness or precipitate will be evaluated visually and using a light microscope. If necessary, the test item stock solution in vehicle will be further diluted (e.g., ½ log lower) to define the highest soluble concentration of test item in the incubation buffer.

8.2.2. TPO-Catalyzed Iodination Assay

In the TPO-catalyzed iodination assay, incubations will be performed with FTC-238-hrTPO cell lysates to determine the possible suppressive effect of the test items on the TPO-catalyzed iodination by measuring the formation of the metabolite MIT. Five independent and valid runs will be performed for each test item. In case of invalid results, a maximum of eight runs will be performed. In case two consecutive invalid runs occur, the Study Director will inform the Sponsor EURL ECVAM. Concentrations of the test item used in a second and/or any subsequent experiments might be adjusted, if necessary.

Incubation mixtures will be prepared on ice by mixing phosphate buffer (0.1 M, pH 7.4), potassium iodide (final concentration 150 µM), L-tyrosine (final concentration 500 µM) FTC-238-hrTPO cell lysate (fixed concentration, details will be specified in the Final Report) and vehicle, test item or control item. After shaking, the samples will be pre-incubated for 5 minutes at 37±1°C in a water bath and the reaction will be started by the addition of 20 µL hydrogen peroxide (final concentration 250 µM). The total volume will be 300 µL. Incubations will be stopped after the appropriate incubation time (fixed time, details will be specified in the Final Report) by transferring the reaction tubes to ice and addition of a half reaction volume of the appropriate IS working solution (fixed IS concentration in the IS working solution, details will be specified in the Final Report). After vortex-mixing of the containers, the samples will be kept on ice until centrifugation (2000 *g* for at least 5 minutes at 4°C) and prepared for MIT analysis by UPLC-MS/MS.

When the five test items are being tested, in total two incubation plates will be prepared. Vehicle controls, assay buffer controls (no vehicle controls), no peroxide controls, non-

enzymatic iodination controls, the negative control DEHP at a single concentration (1 mM) and incubations with the appropriate test items will be included on each incubation plate in triplicate. The first incubation plate will also contain the reference item PTU which will be tested at eight concentrations (1 nM, 10 nM, 100 nM, 316 nM, 1 µM, 3.16 µM, 10 µM and 31.6 µM) in triplicate. The second incubation plate will also contain the reference item PTU which will only be tested at the highest concentration (31.6 µM) in triplicate.

An overview of the incubations included for each independent experiment is presented in the table below.

Composition of Incubations for Each Independent Experiment

Constituent (final concentration)	No peroxide control	Vehicle control	No vehicle control	Non-enzymatic iodination control	Test item, reference item or negative control item incubations
Phosphate buffer (0.1 M, pH 7.4)	X	X	X	X	X
Tyrosine (500 µM)	X	X	X	X	X
Potassium Iodide (150 µM)	X	X	X	X	X
H_2O_2 (250 µM)	---	X	X	X	X
FTC-238-hrTPO cell lysate	X	X	X	---	X
Inactivated FTC-238-hrTPO cell lysate	---	---	---	X	---
Vehicle (1%)	X	X	---	X	---
Test item or PTU (in triplicate) and DEHP (in triplicate)	---	---	---	---	X
Number of incubations	6	6	6	6	153

At least five independent experiments will be performed. Concentrations of the test items used for a second and/or any subsequent experiments may be adjusted, if necessary.

8.3. Analysis of MIT

MIT and IS peak areas in the samples will be measured by UPLC-MS/MS using the method validated in Charles River Test Facility Study No. 20278296.

8.3.1. MIT Stock and Spiking Solutions

For analytical run, duplicate MIT stock solutions (stocks A and B) will be prepared at a 10 mM concentration in 0.1 M HCl in glass vials. Stock solutions will be prepared freshly on the day of use and any residual volumes will be discarded unless otherwise requested by the Study Director.

MIT stock solutions will be diluted in 0.1 M potassium phosphate buffer pH 7.4 to obtain spiking solutions for the preparation of calibration standards and quality control (QC) samples. Spiking solutions will be prepared freshly for each analytical run as presented in the tables below.

Preparation of MIT Spiking Solutions used for the Preparation of Calibration Standards

Code	Applied solution	Volume applied (µL)	Volume buffer added (µL) [1)]	Target concentration (µM)
Spike 0.0761 µM	Spike 0.352 µM	108	392	0.0761
Spike 0.163 µM	Spike 0.756 µM	108	392	0.163
Spike 0.352 µM	Spike 1.63 µM	108	392	0.352
Spike 0.756 µM	Spike 3.50 µM	108	392	0.756
Spike 1.63 µM	Spike 7.55 µM	108	392	1.63
Spike 3.50 µM	Spike 16.2 µM	108	392	3.50
Spike 7.55 µM	Spike 35.0 µM	108	392	7.55
Spike 16.2 µM	Spike 75.0 µM	108	392	16.2
Spike 35.0 µM	150 µM – B	116.5	383.5	35.0
Spike 75.0 µM	150 µM – A	300	300	75.0
150 µM – B	Stock B	15	985	150
150 µM – A	Stock A	15	985	150

[1)] The buffer will consist of 0.1 M potassium phosphate buffer pH 7.4.

Preparation of MIT Spiking Solutions used for the Preparation of QC Samples

Code	Applied solution	Volume applied (µL)	Volume buffer added (µL) [1)]	Target concentration (µM)
Spike 15 µM	Spike 75 µM	200	800	0.150
Spike 75 µM	Spike 600 µM	125	875	0.750
Spike 600 µM	Spike 6 mM	100	900	6.00
Spike 6 mM	Stock A	600	400	60.0

[1)] The buffer will consist of 0.1 M potassium phosphate buffer pH 7.4.

If applicable, other volumes may be used as long as the ratios between components remain the same.

8.3.1.1. Calibration Standards

Ten calibration standards will be prepared from MIT spiking solutions which will be prepared from two MIT stock solutions (A and B as described in Section 8.3.1). An aliquot of the appropriate spiking solution will be added to a 3000 ng/mL IS working solution as described in the table below. Samples will be vortex-mixed and prepared for UPLC-MS/MS analysis by diluting them 100-fold in phosphate buffer (0.1 M, pH 7.4). Calibration standards will be prepared freshly on the first day of use.

Preparation of Calibration Standards in Plasma

Code	Applied solution	Volume Applied		Target concentration (µM) [2]
		Spiking solution (µL)	IS Working Solution (µL)	
MIT 0.0 µM	Buffer [1]	300	150	0
MIT 0.0761 µM	Spike 0.0761 µM	300	150	0.0761
MIT 0.163 µM	Spike 0.163 µM	300	150	0.163
MIT 0.352 µM	Spike 0.352 µM	300	150	0.352
MIT 0.756 µM	Spike 0.756 µM	300	150	0.756
MIT 1.63 µM	Spike 1.63 µM	300	150	1.63
MIT 3.50 µM	Spike 3.50 µM	300	150	3.50
MIT 7.55 µM	Spike 7.55 µM	300	150	7.55
MIT 16.2 µM	Spike 16.2 µM	300	150	16.2
MIT 35.0 µM	Spike 35.0 µM	300	150	35.0
MIT 75.0 µM	Spike 75.0 µM	300	150	75.0

[1] The buffer will consist of 0.1 M potassium phosphate buffer pH 7.4.
[2] Concentration in the sample before addition of the IS working solution (see Section 6.1.4).

If applicable, other volumes may be used as long as the ratios between components remain the same.

8.3.1.2. Preparation of Quality Control (QC) Samples

Spiking solutions of the test item will be applied to prepare quality control (QC)-0, low (L), -middle (M), -high (H) samples. First, a blank matrix working solution will be prepared by mixing the following components:

(1) 4631 µL 0.1 M potassium phosphate buffer pH 7.4
(2) 300 µL 10 mM L-Tyrosine dissolved in 0.1 M HCl
(3) 9 µL 100 mM potassium iodide prepared in buffer (1)
(4) 600 µL 50 µg/mL of heat-inactivated FTC-238-hrTPO cell lysate solution in buffer (1)

An aliquot of the appropriate spiking solution will added to the blank matrix working solution as described in the table below. The QC samples will prepared freshly on the first day of use.

Preparation of the Test Item QC-L QC-M and QC-H Samples in Dialysis Buffer

Code	Applied solution	Volume Applied			Target concentration (µM) [2]
		Spiking solution (µL)	Blank Matrix Working Solution (µL)	IS Working Solution(µL)	
QC-0	Buffer [1]	3	277	150	0
QC-L	Spike 15 µM	3	277	150	0.150
QC-M	Spike 75 µM	3	277	150	0.750
QC-H	Spike 6 mM	3	277	150	60.0

[1] The buffer will consisted of 0.1 M phosphate buffer pH 7.4.
[2] Concentration in the sample before addition of the IS working solution (see Section 6.1.4).

QC-0, QC-L, QC-M and QC-H samples will be prepared in polypropylene tubes in triplicate as described in the table above and will be vortex-mixed after which 3.75 µL of H_2O_2 will be added to each tube. After vortex-mixing of the containers one by one, the samples will be kept on ice until centrifugation (2000 *g* for at least 5 minutes at 4°C) and prepared for UPLC-MS/MS analysis by diluting them 100-fold in potassium phosphate buffer (0.1 M, pH 7.4).

If applicable, other volumes may be used as long as the ratios between components remain the same.

8.3.1.3. UPLC-MS/MS Analysis

MIT and IS peak areas in the samples will be measured by UPLC-MS/MS using the following system:

- Acquity UPLC I-Class system (Waters, Milford, MA, USA)
- Xevo TQ-S mass spectrometer (Waters)

Data will be acquired and interpreted with MassLynx software (Waters).

9. ACCEPTANCE CRITERIA

9.1. Sample Analysis

An UPLC-MS/MS analytical run is considered acceptable if the criteria for the calibration curve and the QC samples are met.

9.1.1. Calibration Curve

The response of the calibration standards will be correlated with the nominal MIT concentration of the calibration solutions using regression analysis with a $1/x^2$ weighting factor. Calibration curves with back calculated accuracies within the criterion range of 80-120% of the nominal concentration for the lowest calibration standard and 85-115% of the nominal concentration for the remaining calibration standards will be accepted.

When a back calculated accuracy (once established) does not comply with the criterion range, the response of the calibration standard with the highest deviation will be rejected and the calibration curve will be re-evaluated. Calibration curves will be accepted when ≥ 75% of the calibration standards fulfill the acceptance criteria.

Zero will not be part of the calibrated range. Blank samples will not be taken into account in the fitting procedure.

9.1.2. QC Samples

The analytical method will be considered applicable for the quantitative analysis of MIT in the samples when the accuracy of the QC samples is in the criterion range of 85-115%. Results outside the criterion range may be discarded as long as per run 2/3 of the QC samples are accepted with ≥ 50% of each level.

9.2. TPO-Catalyzed Iodination Assay

An independent TPO-catalyzed iodination experiment will be considered acceptable if the following criteria are met:

- The final curve for the reference item PTU is composed of a minimum of six concentrations obtained from the average of three replicates after excluding samples on the basis of insolubility, operator errors or other information.
- The final curve for a test item is composed of a minimum of six concentrations obtained from the average of three replicates after excluding samples on the basis of insolubility, operator errors or other information.

- The percent TPO-catalyzed iodination of the lowest test item concentration is within the range of 80%-120% when compared to the average activity in the vehicle control samples.
- A complete sigmoidal curve for the reference item PTU is obtained.
- The calculated IC_{50} for PTU is within the range of 5×10^{-7}-5×10^{-6} M.
- The percent TPO-catalyzed iodination compared to the vehicle control for the negative control DEHP is > 80%.
- The percent TPO-catalyzed iodination in each of the individual no-peroxide control samples is < 1% when compared to the average activity in the vehicle control samples.
- The mean percent TPO-catalyzed iodination of the no-vehicle controls is within the range of 80%-120% when compared to the average TPO-catalyzed iodination in the vehicle control samples.

9.3. Data Interpretation Criteria

For each run, a test item will be considered negative when the percentage of TPO-catalyzed iodination compared to the average activity in the vehicle control samples does not exceed 80% for any concentration.

For each run, a test item will be considered positive when the percent of TPO-catalyzed iodination compared to the average activity in the vehicle control samples is less or equal to 80% for at least one concentration and is showing a dose-dependent effect.

A run will be considered inconclusive in all other cases.

If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the study, the test will be rejected and repeated.

10. ANALYSIS

10.1. MIT Analysis

Response (R)

Peak area of the analyte × (IS Conc./ IS peak area) [units]

Calibration curve

$R = a \times C_N + b$

where:

a = linear regression factor

b = intercept

C_N = nominal concentration

Regression analysis will be performed using the least squares method.

Analyzed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \text{ [µM]}$$

Please note that if a different dilution factor for the study samples has been used than the 100-fold dilution factor used for the calibration standards and QC samples, MIT concentrations of the study samples need to be corrected for this. For example, if a 20-fold dilution factor was used for the study samples (and a 100-fold dilution factor for the calibration standards and QC samples), the calculated MIT concentration in the study samples needs to be divided by a factor 5.

Accuracy of analytical QC samples

$$\frac{C_A - C_B}{C_N} \times 100 \text{ [\%]}$$

where:

C_B = analyzed concentration in QC-0 sample

10.2. TPO-Catalyzed Iodination

Calculate the percent of TPO-catalyzed iodination compared to the average TPO-catalyzed iodination in the vehicle control samples (= full activity) for each individual sample (vehicle control, no vehicle control, no peroxide control, DEHP, PTU and test item samples) using the following equation:

$$\%\text{TPO catalyzed iodination} = \frac{C_A \text{ in sample}}{\text{Average } C_A \text{ of vehicle control samples}} \times 100\%$$

If applicable, the IC_{50} value will be calculated by plotting the percentage of control activity versus the logarithm of the concentration fitted by the Hill curve model (variable slope, 4 parameters) using GraphPad Prism (GraphPad Software, San Diego, USA) and the following equation:

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{\left(1 + 10^{((\text{Log}IC_{50} - x) * \text{HillSlope})}\right)}$$

In which the variables will be defined as follows:

Y	= Percent of the control activity
X	= Logarithm (base 10) of the concentration
Top	= Top of the curve in same units as Y
Bottom	= Bottom of the curve in same units as Y
Log IC_{50}	= Logarithm of concentration at which 50% of maximum response is observed
HillSlope	= Slope factor of the Hill curve

11. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
Deviation Information Library	Deviations
M-Files®	Reporting and collection of 21 CFR Part 11 compliant signature
Magellan Tracker	Optical Density Measurement System control and data acquisition
MassLynx	System control, data acquisition and integration
REES Centron	Temperature, relative humidity and/or atmospheric pressure monitoring

Data for parameters not required by Study Plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by Study Plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

12. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

13. QUALITY ASSURANCE

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the Study Plan, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

14. AMENDMENTS AND DEVIATIONS

Changes to the approved Study Plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary Study Plan changes in advance with the Sponsor. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible. In addition, in case there are two consecutive invalid runs, the Study Director will inform the Sponsor about that.

15. RETENTION AND DISPOSITION OF RECORDS AND SAMPLES

All applicable study-specific raw data, electronic data, documentation, Study Plan and Final Report will be archived by no later than the date of Final Report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least 2 years after issue of the Final Report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study Plan, Study Plan amendments, and deviations
- Study schedule
- Study-related correspondence

- Test system receipt
- Test item receipt, identification and preparation
- Measurements and observations

A verified copy of the applicable study-specific raw data will be sent to the Sponsor at the following location:

European Commission (DG-JRC)
Directorate F - Health, Consumers and Reference Materials
Unit F3 - Chemical Safety and Alternative Methods / The European Union Reference Laboratory for Alternatives to Animal Testing
(EURL ECVAM)
Via E. Fermi, 2749. TP126
I-21027 Ispra (VA)
Italy

Disposition of residual/retained analytical samples will be as described in the table below.

Disposition of Residual/Retained Samples

Sample Type	Disposition	Schedule
Analytical (and Test Item used in analysis)	Discard	After completion of the measurements

16. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable). The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

17. JUSTIFICATIONS AND GUIDELINES

17.1. Guidelines for Study

This study is not within the scope of regulations governing the conduct of nonclinical laboratory studies and is not intended to comply with such regulations.

18. REFERENCES

1. Doerge, D.L., Chang, H.C., Divi, R.L., Churchwell, M.I., Mechanism for inhibition of thyroid peroxidase by Leucomalachite Green. Chemical Research in Toxicology 11, p. 1098-1104 (1998).

2. Freyberger, A., Ahr, H.-J., Studies on the goitrogenic mechanism of action of *N,N,N'N'*-tetramethylthiorea. Toxicology, p. 169-175 (2006).

3. Price, R.J., Burch, R., Chatham, L.R., Higgins, L.G., Currie, R.A., Lake, B.G., An assay for screening xenobiotics for inhibition of rat thyroid gland peroxidase activity. Xenobiotica, DOI: 10.1080/00498254.2019.1629044.

TEST FACILITY APPROVAL

All electronic signatures appear at the end of the document upon finalization.

SPONSOR APPROVAL

The Study Plan was approved by the Sponsor by e-mail on the date designated below. The correspondence giving approval will be archived, as appropriate with other Sponsor communications.

12 Aug 2021
Date of Sponsor Approval

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	Recipient
Original	Study Director
1 Copy	Radiation Safety Officer
1 Copy	Sponsor Representative / Study Monitor
1 Copy	QAU / Management

SIGNATURE(S) FOR DOCUMENT: 20309164 - 212555 212556 212557 212559 RS Validation of TPO inhibition assay Final Study Plan

Testing Facility Management Approval-NGLP: I approve the Study Director identified in this document and acknowledge the study.

Name: **Wenker, Mira**

Wenker, Mira

30-Aug-2021 09:52:17 (UTC+00:00)

Electronically Signed in M-Files Timestamp

Study Director Approval: I approve this document.

Name: **Reinen, Jelle**

Reinen, Jelle

30-Aug-2021 09:55:06 (UTC+00:00)

Electronically Signed in M-Files Timestamp

Appendix 3
Test Item Characterization

Certificate of Analysis of Flavanone

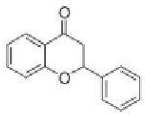
SIGMA-ALDRICH® sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Flavanone - 98%

Product Number:	**102032**
Batch Number:	**MKCG6841**
Brand:	ALDRICH
CAS Number:	487-26-3
MDL Number:	MFCD00006841
Formula:	C15H12O2
Formula Weight:	224.25 g/mol
Quality Release Date:	19 JUN 2018



Test	Specification	Result
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder or Crystals	Powder
Infrared Spectrum	Conforms to Structure	Conforms
Purity (HPLC)	≥ 97.5 %	100.0 %

Michael Grady, Manager
Quality Control
Milwaukee, WI US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

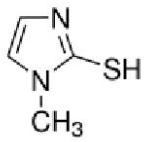
Certificate of Analysis of Methimazole

SIGMA-ALDRICH® sigma-aldrich.com
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
2-Mercapto-1-methylimidazole - ≥99%

Product Number:	**301507**
Batch Number:	**WXBC8588V**
Brand:	ALDRICH
CAS Number:	60-56-0
Formula:	C4H6N2S
Formula Weight:	114.17 g/mol
Quality Release Date:	19 OCT 2018



Test	Specification	Result
Appearance (Colour)	White to Off-White	Off-White
Appearance (Form)	Powder or Crystalline Powder or Crystals or Granules	Powder
Infrared Spectrum	Conforms to Structure	Conforms
Purity (GC)	≥ 99.0 %	99.7 %
Carbon	41.6 - 42.5 %	41.8 %
Nitrogen	24.3 - 24.8 %	24.7 %

Steven Chen, Manager
Quality Control
Wuxi, China CN

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

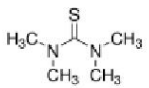
Certificate of Analysis of N,N,N,N-tetramethylthiourea

SIGMA-ALDRICH® sigma-aldrich.com
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Tetramethylthiourea – 98%

Product Number:	**115169**
Batch Number:	**SHBJ4707**
Brand:	ALDRICH
CAS Number:	2782-91-4
MDL Number:	MFCD00008324
Formula:	C5H12N2S
Formula Weight:	132.23 g/mol
Storage Temperature:	Store at 2 - 8 ℃
Quality Release Date:	02 OCT 2017



Test	Specification	Result
Appearance (Color)	White to Yellow	White
Appearance (Form) Crystals or Crystalline Powder and/or Chunks	Conforms to Requirements	Powder
Infrared Spectrum	Conforms to Structure	Conforms
Carbon	44.3 - 46.5 %	45.3 %
Nitrogen	20.7 - 21.7 %	21.1 %
Sulfur (S)	23.6 - 24.9 %	24.2 %
Purity (TLC)	≥ 98 %	100 %
Solubility (Turbidity)	Clear	Clear
Solubility (Color) Solubility Concentration: 5% in 95% Ethanol	Colorless to Very Faint Yellow	Colorless

Michael Grady, Manager
Quality Control
Sheboygan Falls, WI US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Certificate of Analysis of Naringenin

Combi-Blocks, Inc
7949 Silverton Ave, Suite 915
San Diego, CA 92126, USA

Toll free: 1-877-5-BLOCKS
International: 1-858-635-8950
Fax: 1-858-635-8991
Email: sales.team@combi-blocks.com
Web Site: www.combi-blocks.com

CERTIFICATE OF ANALYSIS

Product Number	**QZ-3964**
Product Name	(2R)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one
CAS Number	480-41-1
Molecular Formula	$C_{15}H_{12}O_5$
Molecular Weight	272.3

TEST RESULTS

BATCH NUMBER	**A63107**
APPEARENCE	White solid
BOILING POINT	No Data
MELTING POINT	No Data
NMR	95% , conform with structure

Howard Zhang, Ph.D.
QC director

01/03/19

Acceptance Date

Certificate of Analysis of Sulfamethazine

SIGMA-ALDRICH®

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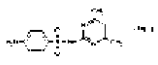
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Sulfamethazine sodium salt - ≥98%



Product Number:	**S5637**
Batch Number:	**WXBD0389V**
Brand:	SIGMA
CAS Number:	1981-58-4
Formula:	C12H13N4NaO2S
Formula Weight:	300,31 g/mol
Storage Temperature:	Store at 2 - 8 °C
Quality Release Date:	17 JUL 2019
Recommended Retest Date:	JUN 2023

Test	Specification	Result
Appearance (Colour)	White to Off White	White
Appearance (Form)	Powder	Powder
Solubility (Colour)	Colorless to Faint Yellow	Colorless
Solubility (Turbidity) 50 mg/mL, H2O	Clear	Clear
Loss on Drying	≤ 6.0 %	0.4 %
% Purity (Titration) Dry Basis	≥ 98	100

Steven Chen, Manager
Quality Control
Wuxi, China CN

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

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QA Approval:	I approve the Quality Assurance Statement for this report.
Name:	**Kock, Marjolijn**
	Kock, Marjolijn
Electronically Signed in M-Files	Timestamp: 30-Jun-2022 11:34:50 (UTC+00:00)

Study Director Approval:	I approve this Report.
Name:	**Reinen, Jelle**
	Reinen, Jelle
Electronically Signed in M-Files	Timestamp: 30-Jun-2022 11:40:45 (UTC+00:00)