

IMI2 GA853989 - ERA4TB

European Regimen Accelerator For Tuberculosis

WP4 – IMAGING

D4.2 Implementation of PK/PD MALDI-MS imaging to analyses infected tissue

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¹ Use one of the following codes: R: Document, report (excluding the periodic and final reports); DEM: Demonstrator, pilot, prototype, plan designs; DEC: Websites, patents filing, press & media actions, videos, etc.; OTHER: Software, technical diagram, etc.

² Please choose the appropriate reference and delete the rest: PU = Public, fully open, e.g. web; CO = Confidential, restricted under conditions set out in Model Grant Agreement.

Definitions

Partners of ERA4TB are referred to herein according to the following codes:

Grant Agreement. The agreement signed between the beneficiaries and the IMI JU for the undertaking of the ERA4TB project.

Project. The sum of all activities carried out in the framework of the Grant Agreement.

Work plan. Schedule of tasks, deliverables, efforts, dates and responsibilities corresponding to the work to be carried out, as specified in Annex I to the Grant Agreement.

Consortium. The ERA4TB Consortium, comprising the legal entities signatories of the Grant Agreement.

Consortium Agreement. Agreement concluded amongst ERA4TB participants for the implementation of the Grant Agreement. Such an agreement shall not affect the parties' obligations to the Community and/or to one another arising from the Grant Agreement.

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3. Abstract

As a relevant part of ERA4TB scientific and technological platforms, Work Package 4 (WP4) (*Imaging*) is focused on the use of cutting-edge imaging techniques to improve the understanding of *Mycobacterium tuberculosis* (Mtb) infection, deliver high value biomarkers to track pre-clinical and clinical evolution of Tuberculosis (TB) and speed up drug discovery process providing unique insights on drugs pharmacokinetic/pharmacodynamic (PK/PD) properties as well as drug distribution in infected tissues.

The concentration and distribution of a drug or its metabolites in tissues are key factors influencing drug efficacy or toxicity. Conventional pharmacokinetic studies show that the plasma concentration of a drug is often not fully translatable to the intra-tissue concentration. Moreover, it is difficult to predict the distribution of a drug in tissues, particularly in those with complex structures like TB lesions.

At that extent, Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry (MS) is a key imaging technology which applied to infected tissues will provide quantitative information about drug penetration and distribution at the site of action (different types of TB lesions). This approach allows to determine biodistribution, accumulation and PK profiles of the drugs without any labelling and preserving the histological integrity of tissues.

As part of WP4 there has been defined a specific task (Task 4.2: Implementation of PK/PD MALDI-MS imaging to analyse infected tissue) aimed to create a robust MALDI-MS platform to better understand the PK/PD behaviour of new drugs in the ERA4TB pipeline, either alone or in combination.

Teams directly involved in this task are ImaBiotech SAS (Loos, France) (IBT) and GlaxoSmithKline I+D S.L (Tres Cantos, Spain) (GSK).

Main work and deliverables for the start of ERA4TB project were focused in implementing MALDI-MS capabilities as a standardized platform able to work with infected Mtb samples that could be generated as part of the activity of ERA4TB either as capacity creation or pipeline progression experiments.

To achieve that target, key action for GSK has been setting-up MALDI-MS platform inside its Biosafety Level 3 (BSL3) facilities in Tres Cantos and the definition of a sterilization workflow for IBT to work with Mtb infected samples outside of biocontainment. In addition, both partners have committed to develop analytical methods for drugs used either as standard of care or being tested in advanced clinical trials. Progress so far is reported in the present Deliverable document.

4. Introduction

Concentration and distribution of a drug or its metabolites in tissues are key factors for understanding drug efficacy and toxicity. Conventional pharmacokinetic studies show that the plasma concentration of a drug is not always a perfect surrogate of the intra-tissue concentration. Moreover, it is difficult to predict the distribution of a drug in tissues, particularly those with complex structures due to infections, as it is the case for *Mycobacterium tuberculosis* lesions. Even though the overall tissue concentration can be measured by using homogenizing procedures, sometimes micro-distribution in the lesions will be critical to have better understanding of the different behaviour of the antimicrobial drugs. Matrix Assisted Laser

Desorption/Ionization (MALDI) Mass Spectrometry Imaging (MSI) is a well-suited technology to address these key questions¹.

5. MALDI Methodology description

MALDI MSI is a molecular imaging technique aiming at probing the molecular content of tissue sections in any point of the sample and restitution this information as an ion density map showing easily the spatial distribution of the molecule of interest in the sample². The use of Mass Spectrometry for molecule identification allows to detect a hugely wide range of molecules and metabolites not requiring any additional labelling technology and has become standard approach in a wide number of biomedical areas^{3,4,5}.

In ERA4TB Consortium, MALDI analysis is being performed at laboratory premises of two partners: GSK I+D in Tres Cantos, Spain and ImaBiotech SAS (IBT) in Loos, France.

i. Principle

MALDI is a soft ionization that involves a laser striking a matrix of small molecules to make the analyte molecules into the gas phase without fragmenting or decomposing them^{6,7}. Some biomolecules are too large and can decompose when heated, and traditional techniques will fragment or destroy macromolecules. MALDI is appropriate to analyse biomolecules like peptides, lipids, saccharides, or other organic macromolecules. Starting historically from biomolecules, the field of application of MALDI MS has now extended to small molecules such as drugs and drug metabolites.

The analyte is embedded in a very large excess of a matrix compound deposited on a solid surface called a target, usually made of a conducting metal and having spots for several different samples to be applied.

¹ Nishidate, M., Hayashi, M., Aikawa, H., Tanaka, K., Nakada, N., Miura, S. I., ... & Hamada, A. (2019). Applications of MALDI mass spectrometry imaging for pharmacokinetic studies during drug development. *Drug metabolism and pharmacokinetics*, 34(4), 209-216.

² Chughtai, K., & Heeren, R. M. (2010). Mass spectrometric imaging for biomedical tissue analysis. *Chemical reviews*, 110(5), 3237-3277.

³ Guerrero, I. C., & Kleiner, O. (2005). Application of mass spectrometry in proteomics. *Bioscience reports*, 25(1-2), 71-93.

⁴ Duncan, M. W., Roder, H., & Hunsucker, S. W. (2008). Quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Briefings in functional genomics and proteomics*, 7(5), 355-370.

⁵ Fuchs, B., & Schiller, J. (2009). Application of MALDI-TOF mass spectrometry in lipidomics. *European Journal of Lipid Science and Technology*, 111(1), 83-98.

⁶ Gross, J. H. (2006). *Mass spectrometry: a textbook*. Springer Science & Business Media.

⁷ Boesl, U. (2017). Time-of-flight mass spectrometry: introduction to the basics. *Mass spectrometry reviews*, 36(1), 86-109.3.

After a very brief laser pulse, the irradiated spot is rapidly heated and becomes vibrationally excited. The matrix molecules energetically ablated from the surface of the sample, absorb the laser energy and carry the analyte molecules into the gas phase as well. During the ablation process, the analyte molecules are usually ionized by being protonated, cationized or deprotonated with the nearby matrix molecules. The most common MALDI ionization format is for analyte molecules to carry a single charge.

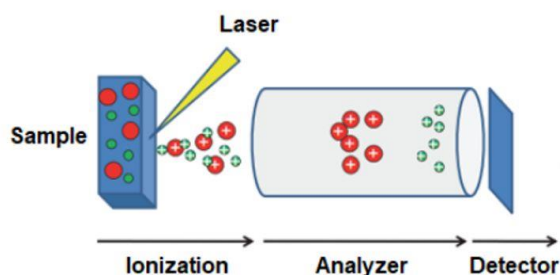


Figure 1: Basic scheme of MALDI technology⁸

ii. Sample preparation

Samples (isolated organ or whole-body rodent) are cryosectioned with a HM360 cryostat (Thermo Fisher, Germany) or Starlet cryostat and mounted on an ITO slide (Delta Technologies, CO, USA). The MALDI matrix is then sprayed homogeneously on top of the slide with an automatic TM sprayer (HTX Technologies, Chapel Hill, NC, USA) and ImagePrep System (Bruker, Germany) as illustrated in Figure 2.

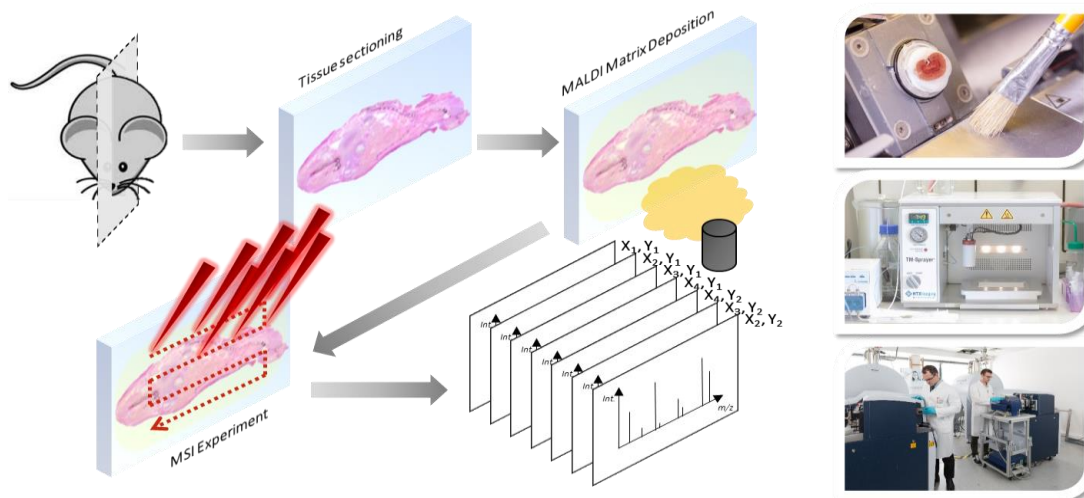


Figure 2: MALDI Mass Spectrometry workflow

⁸ https://en.wikipedia.org/wiki/Matrix-assisted_laser_desorption/ionization

A calibration curve can be prepared and spotted on control tissue sections (IBT's method) or using the MIMETIC approach (GSK's method). Options are graphically Presented in Figure 3.

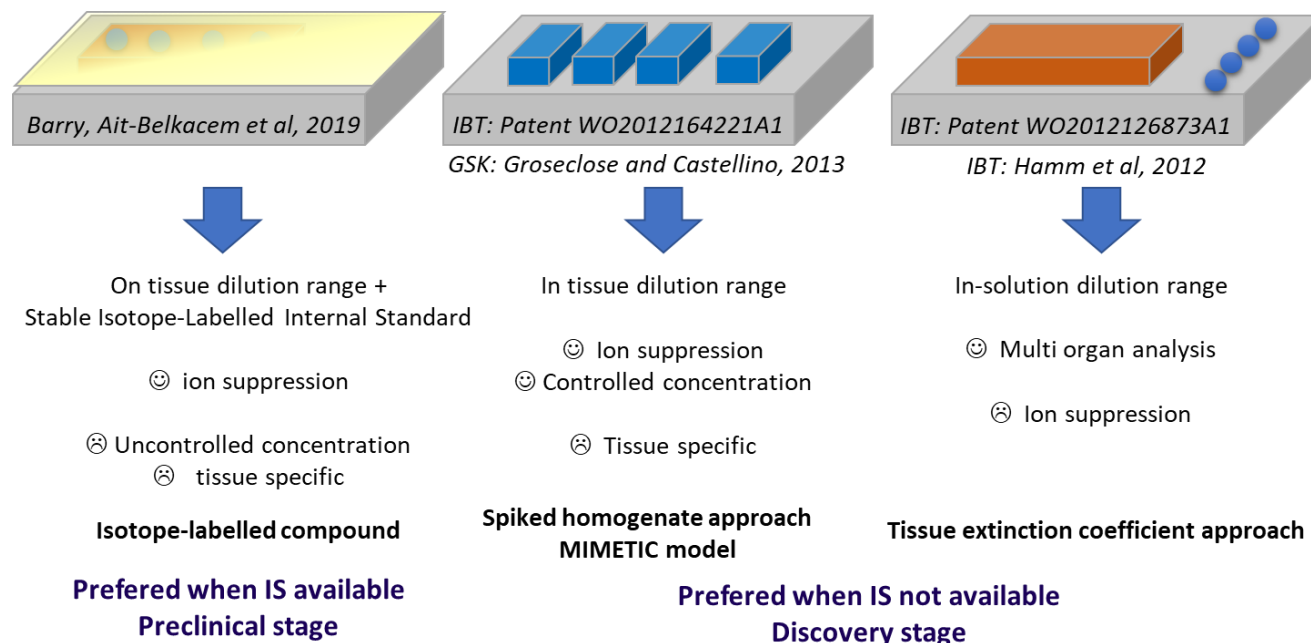


Figure 3: Quantitative Mass Spectrometry Imaging Workflows

iii. Mass Spectrometry

The prepared slides are introduced in the MALDI Mass Spectrometer 7T or Solarix or UltrafleXtreme (Bruker Daltonics, Bremen, Germany) at IBT and GSK, respectively. A Smartbeam II laser shoots in every position of the tissue and generates an ion cloud in the gas phase which is then analysed, either by Fourier-Transformed Ion Cyclotron Resonance (FT-ICR – Solarix) or Time of Flight (TOF - UltrafleXtreme).

iv. Data Analysis and Quantification

The distribution of a drug or any exogenous or endogenous molecule can be represented as a color-coded ion density map and allow to extract qualitative information on the distribution of the molecule in the tissue.

Thanks to the calibration curves built using one of approaches illustrated in Figure 3, a quantification in $\mu\text{g/g}$ or μM in tissue can be generated for each region of interest (e.g. whole lung, airways, caseum, etc). These concentrations will be usable for simple tissue pharmacokinetics (PK) interpretation, or further by inclusion of these data in more complex models such as physiologically based pharmacokinetics (PBPK) models with the contribution of WP5.

6. MALDI Deployment in ERA4TB. Requirements and established Objectives.

The suitability of MALDI technology to generate relevant information about drug distribution at target level, interactions in drug combinations and translational preclinical PK/PD was clear from the ERA4TB project inception. The value of MALDI is particularly relevant for TB for which the pathophysiology of lesions induced by the infection can much influence the drug (or drug combination) disposition and access to the different populations of the target microorganism.

Whereas there are a number of options to evaluate compound distribution in non-infected preclinical models, capability to perform these measurements in TB infected samples is hampered by biosafety constraints. However, maximum value of MALDI readouts will be obtained when applied to infected tissues and biological samples. It is then required to define, set up and validate procedures that ensure that the work with the infected tissues or samples is safe whilst maintaining biological material's integrity and not modifying compound's amount or spatial distribution.

Two parallel strategies have been considered to allow MALDI work with TB infected samples:

- Deployment of MALDI and enabling associated technologies (i.e. cryosectioning) inside BSL3 containment facilities. This approach has been followed by GSK.
- Selection, validation and logistic procedures development for sterilization techniques applied to biological samples infected with Mtb that allow further safe manipulation for MALDI in BSL2 or BSL1 Lab conditions. This is being the strategy led by ERA4TB partner IBT.

In addition to the workflows related with infected samples, the contribution to MALDI MSI activities of two different institutions (GSK, IBT) requires harmonization of procedures, protocols and data outputs to ensure robustness of results at Consortium level.

In conclusion, the requirements identified to deploy a robust MALDI MSI platform for ERA4TB translate to three Objectives whose level of achievement is reflected in the present Deliverable document:

Objective 1: Installation of a MALDI Imaging resource in BSL3 containment (GSK).

Objective 2: Evaluate and deploy sterilization approaches for IBT.

Objective 3: Set up and harmonization of MALDI MSI methods between GSK and IBT.

7. Implementation status

Objective 1: Installation of a MALDI Imaging resource in BSL3 containment (GSK)

MALDI platform in Tres Cantos is part of Discovery DMPK Organization of GSK and works closely with Global Bioimaging Department in the Company. GSK Global health facilities in Spain include a BSL3 facility to work *in vitro* and *in vivo* with different pathogens, specifically with Mtb. For *in vivo* experiments, available mouse models include BALB/C (acute and chronic) and C3HeB/FeJ (Kramnik) model. Within this facility a room with more than 15 squares meters has been dedicated exclusively for MALDI work. The spaces have been conditioned to avoid any potential Mtb spread in the process of sample manipulation. All activities (and corresponding equipment) able to generate aerosols are enclosed in Biosafety cabinets, one for the cryosection and another one to spray the samples and preparation for MALDI. Graphical description of the MALDI BSL3 premises is shown in Figure 4.

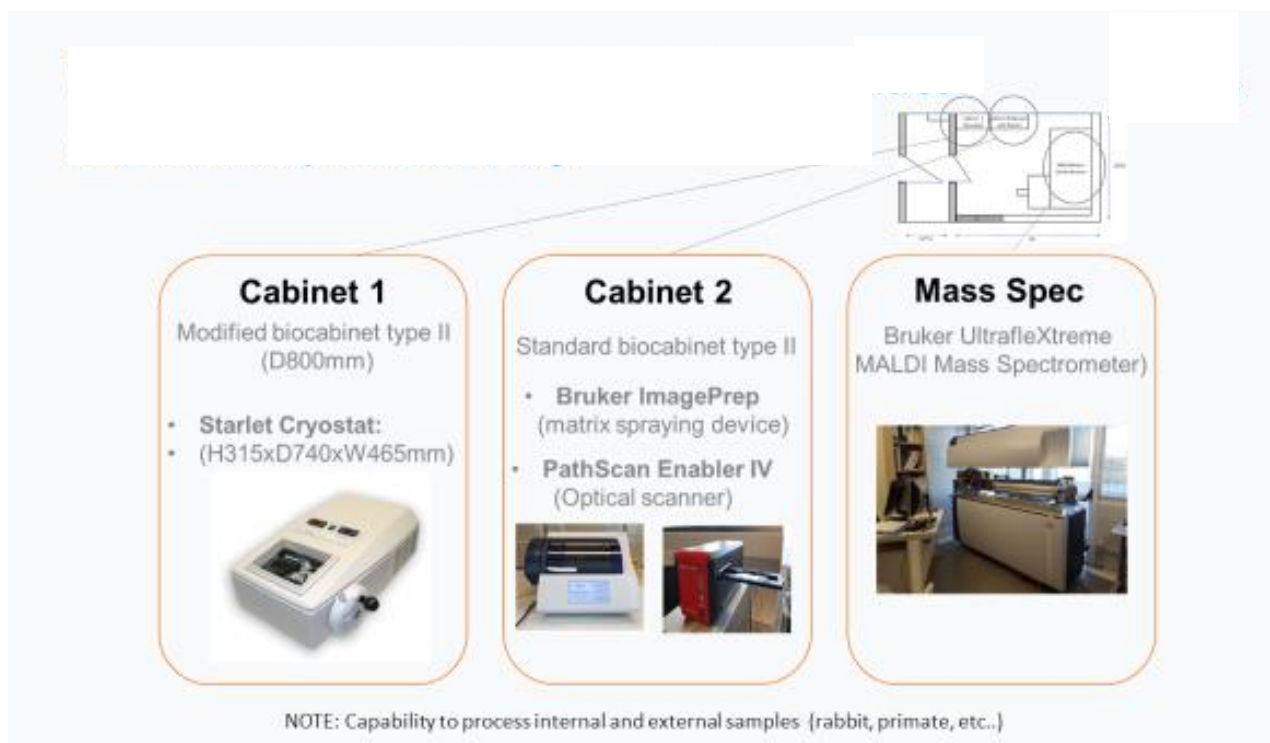


Figure 4: MALDI MSI setting in GSK's BSL3 facility

The MALDI BSL3 suit in GSK is fully operative from the last quarter of 2021. GSK in Tres Cantos has also experience and resources to combine standard PK readouts, both in blood/plasma and in tissue homogenates of infected animals with MALDI results to generate a rich dataset of compounds' pharmacokinetics and distribution.

Objective 2: Evaluate and deploy sterilization approaches for IBT

As IBT's imaging platform is not equipped to work on BSL3 pathogens, a sterilization approach for the biological samples prior their evaluation by MALDI has been sought in order to comply with the mandatory protection toward infectious agents.

This sterilization strategy should destroy *Mtb* cells while preserving histological and molecular integrity of the sample. Considering the various partners involved in generating infected samples within the ERA4TB Consortium, logistical and practical aspects should also be taken into consideration to streamline transfer of samples between sites.

A first step of these investigations has been to determine possible options meeting the above-mentioned criteria and the second step has been to identify potential partners – within or outside the consortium - having the possibility to provide support. It is also assumed in all cases that *ad hoc* tests will have to be carried out to validate the sterilization efficiency, compatibility with MSI requirements and setting-up the sample transfer logistics between sites.

i. Gamma Irradiation

Gamma irradiation using ^{60}Co or ^{137}Cs sources has long been well established as a sterilization technique. The technique allows the sterilization of isolated organs and not only cryosections. The ability to sterilize whole-body mice is still uncertain.

The main issue identified so far for application of irradiation approach is the limited number of facilities with appropriate authorizations, equipment and adequate throughput to cover the Consortium workload. The volume of the sterilization chamber and its ability to be maintained at low temperature with dry ice to keep the sample frozen during the long irradiation times are also concerning topics.

While investigating this technique, Research Center Borstel (FZB) has contributed to the discussion by presenting to WP4.2 members the capabilities of their platform of gamma irradiation using ^{137}Cs . Although their facility would be able to run a pilot test, its limited throughput makes it difficult to consider this partner the preferred option for regular sterilization of samples to be analysed at IBT.

Even accounting for the limitations mentioned, Borstel support and experience will be considered for punctual interventions and, as the technique is still interesting to meet the WP requirements, activities to identify suitable partners, possibly outside of the consortium, will be kept on-going.

ii. Heat Sterilization and Stabilization of organs with Stabilizor™

The Stabilizor™ (<https://denator.com/products/stabilizor-system>) is a device proposed by the Company Denator (<https://www.denator.com/>) offering a similar approach than HSSC (see next section down below) but claimed to be applicable not only to slides but also to isolated organs. It requires no specific skills to be operated hence more flexibility for the animal facilities than the HSSC which requires staff trained to both BSL3 practices and histological preparations.

A contact with University of Uppsala (Prof. Per Andren) has been established since they have an instrument outside a BSL3 facility. However, despite direct and indirect attempts to contact the company to set-up a pilot study together with Institute Pasteur Lille (IPL) which would have provided the samples to be sterilized, it has not been possible to identify a suitable workflow. Validation is also a critical step to ensure technology works properly for TB as this has not been previously tested.

Despite perceived advantages, unfortunately translation to real life practice for the ERA4TB Consortium does not seem possible in the absence of reliable support from Denator. No further efforts will be made in this direction.

iii. Heat Sterilization and Stabilization of Cryosections (HSSC)

The HSSC approach has been proposed by Veronique Dartois's group and recently described in Wang *et al*, 2021⁹. It requires the sectioning of the samples in a BSL3 facility, mounting the sections on slides and heating the slides at 100°C for 1h with a hybridizer or similar device. The resulting sample has been proven to be compatible with the requirements of MALDI MSI.

A preliminary assessment at IBT confirmed this finding at the histological level as can be seen in Figure 5. Histological structures look very similar after mild (84°C) and harsh (100°C) heating conditions.

⁹ Wang, N., Sarathy, J. P., Zimmerman, M., Kaya, F., Wang, H., Dartois, V., & Carter, C. L. (2021). On-Slide Heat Sterilization Enables Mass Spectrometry Imaging of Tissue Infected with High-Threat Pathogens Outside of Biocontainment: A Study Directed at *Mycobacterium tuberculosis*. *Journal of the American Society for Mass Spectrometry*, 32(11), 2664-2674.

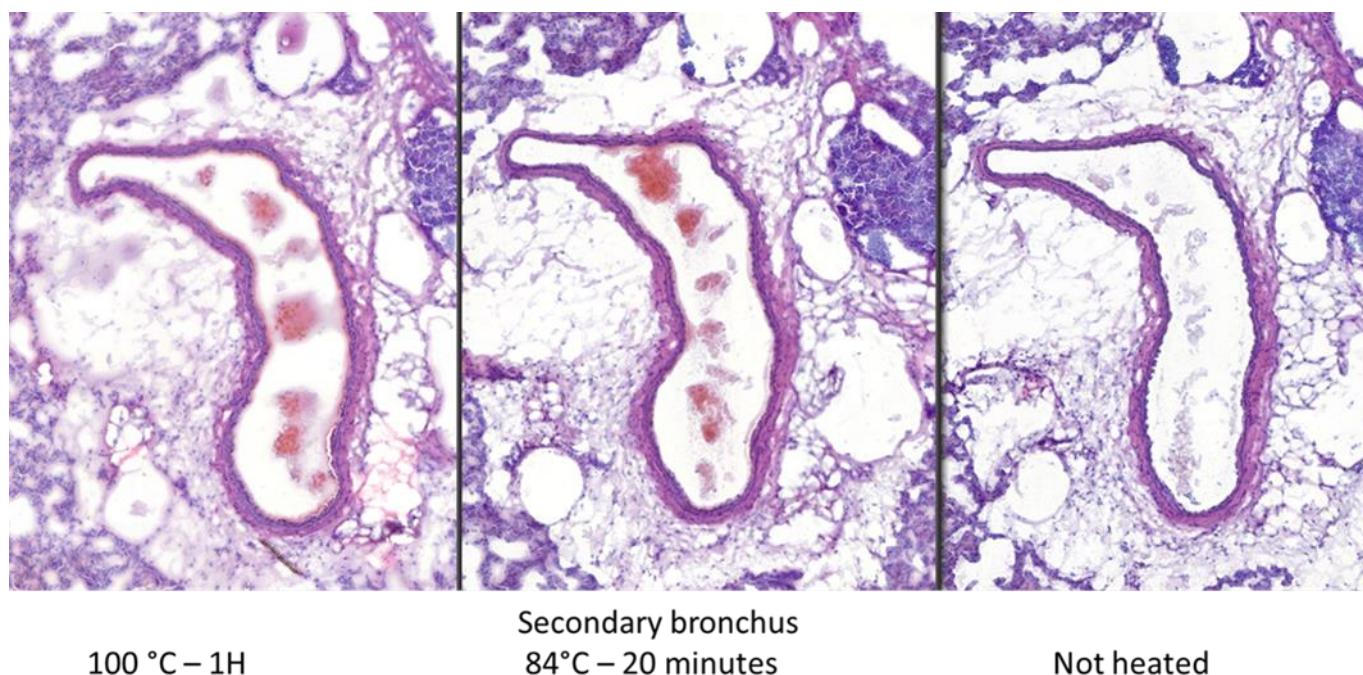


Figure 5: Preliminary results of histological integrity investigation at IBT for Heat Sterilization and Stabilization of Cryosections

The advantage of the approach is its ease of implementation in a BSL3 (small footprint of the hybridizer). Its main drawbacks are the requirement of having sectioning capabilities in a BSL3 facility. Currently, a relevant number of ERA4TB partners generating TB infected samples (GSK, IDMIT, FZB) have this capability although not all.

iv. High Hydrostatic Pressure Sterilization (HHPS)

HHPS is well known technology in the food industry. A contact has been established by IPL with Marie de Lamballerie (GEPEA – UMR CNRS 6144, ONIRIS Veterinary School, Nantes, France). Their team has a suitable equipment with large volume compatible with all samples of interest for the Consortium (isolated rodent and NHP lungs and mouse whole-body).

A pilot study organized with IPL using BCG as a model class 1 microorganism has been performed (4Q2021) with promising results. Sterilization conditions (time versus pressure) require additional optimization. Also, the effect of the HHPS process on the histological structures and compounds' stability and distribution must be evaluated. In addition, the impact of freeze-thaw cycle on the sample will need to be investigated in terms of histological integrity but also possible degradation and delocalization of the drugs.

Despite the number of points still to be addressed, HHPS approach is considered promising and likely the most efficient option to attend the Consortium needs in logistical terms. For these reasons, this strategy will continue to be evaluated during 2022 and 2023.

v. Current status on sterilization strategy

Once the Stabilizer™ approach has been discarded, accounting its versatility, HHPS strategy would be appropriate to allow IBT to support the Consortium efforts on infected samples. However, several points such as sterilization efficiency on TB, histological and molecular integrity still need to be assessed and

properly validated. This work will continue with the support of IPL during 2022 and 2023 and any progress as well as final decisions on application will be shared as an update of this Deliverable 4.2.

At present, HSSC is the strategy that offers the best guaranties of applicability. Different institutions able to generate samples for MALDI have started equipment deployment (IDMIT, FZB). Main setback, namely its logistical implications (cryostat and trained-staff in BSL3 plus hybridizer or similar heater device availability) will be discussed to establish a complete workflow between the partners.

Gamma Irradiation option in FZB will be used for samples generated at the institution and will be sought as backup option to be used in case there's an urgent requirement of MALDI analysis from the ERA4TB Consortium before the final workflow is in place.

As a conclusion, although there is not a centralized, single option defined, the Consortium is able to assume MALDI work with infected samples if requested according to a pipeline progression need.

Objective 3: Set up and harmonization of MALDI MSI methods between GSK and IBT

i. Selection of standards

A selection of standard anti-TB compounds has been done considering those being part of combinations or treatment regimen already in clinical use (standard of care) or at least being tested in advanced clinical trials. This choice is based on the fact that these compounds would most probably be constituents of the backbone of any future drug combination to treat TB so information about them is relevant by itself in anticipation of being combined with candidates hopefully coming from ERA4TB pipeline.

The combinations/regimens in clinical use or in clinical trial considered are:

- Standard of care for Drug Sensitive (DS) TB (HRZE)
- SimpliciTB clinical trial (BPamZ)
- TBTC Study 31 (RptHZE) & (RptHQM)

Based on that the selection of combinations/regimens the list of drugs to standardize the procedures and generate a robust set of data are:

- Isoniazid (H)
- Rifampicin (R)
- Pyrazinamide (Z)
- Ethambutol (E)
- Bedaquiline (B)
- Pretomanid (Pa)
- Moxifloxacin (M)
- Rifapentine (Rpt)

Work performed has focused in determining the Lower Limit of Quantification (LLOQ) and also the Upper Limit (ULOQ), thus defining the linear range MS signal versus Concentration for each compound. Work has been performed initially with *in vitro* samples and afterwards checked with *in vivo* samples (non-infected in all cases).

ii. IBT results

IBT has tested all the above-mentioned compounds in order to develop an MSI method for imaging each of them separately. In a second step, likely in 2022 depending of sample requests, multiplexing of the methods will be considered to streamline the imaging process of samples treated with the combination therapies (HRZE, BPamZ, RptHZE).

Table 1 : Summary of MALDI MSI method performances at IBT

Compound	Short Name	Matrix	Composition	LLOQ (µg/g)	ULOQ (µg/g)
Isoniazid	H	tbd	tbd	tbd	tbd
Rifampicin	R	pNA 5mg/mL 9AA 10 mg/mL	3:1 Acetone:Water 90/10 IPA/Water	8.7 23.4	422 269
Pyrazinamide	Z	DHB 40 mg/mL	1:1 MeOH:Water + 0.1% TFA	397	tbd
Ethambutol	E	DHB 40 mg/mL	1:1 MeOH:Water + 0.1% TFA	13.8	735
Bedaquiline	B	DHB 40 mg/mL	1:1 MeOH:Water + 0.1% TFA	4,3	776
Pretomanid	Pa	DHB 40 mg/mL	1:1 MeOH:Water + 0.1% TFA	11,8	403
Moxifloxacin	M	DHB 40 mg/mL	1:1 MeOH:Water + 0.1% TFA	34,4	2393
Rifapentin	Rpt	9AA 10 mg/mL	90/10 IPA/Water	5,3	300

tbd: to be determined; **pNA**: para-Nitroaniline; **9AA**: 9-Aminoacridine; **DHB**: 2,5-dihydroxybenzoic acid; **IPA**: Isopropyl Alcohol; **TFA**: Trifluoroacetic Acid; **MeOH**: Methanol

As a conclusion of this method development part, anti-TB drugs have very different analytical behaviour and in the best case a limit of detection in the low µg/g range (Table 1). Most of them are ionized with the same sample preparation, using DHB as a MALDI matrix which will be a significant advantage for future imaging studies of combination therapies. However, Rifampicin and Rifapentine have a distinct ionization in negative ion mode.

At this stage of method development, further efforts need to be made to improve Isoniazid, Rifampicin and Pyrazinamide methods, for two reasons. First, they are the poorest method performance, second their place is key in the current therapies.

iii. GSK results

GSK has tested the same compound with different instruments and different analytical method in order to see how it correlates with IBT results. In Table 2, a summary of the current studies performed in vitro to determine the low limit of quantification is presented.

Table 2 : Summary of MALDI MSI method performances at GSK

Compound	Short Name	Matrix	Composition	LLOQ (µg/g)	ULOQ (µg/g)
Isoniazid	H	HCA 7 mg/mL	3:1 EtOH:Water + 0.2% TFA	nd	tbd
Rifampicin	R	Ph-CCA-NH2 5 mg/mL	3:1 ACN:Water	12	292
Pyrazinamide	Z	HCA 7 mg/mL	3:1 EtOH:Water + 0.2% TFA	187	900
Ethambutol	E	DHB 25 mg/mL	1:1 MeOH:Water + 0.2% TFA	2	50
Bedaquiline	B	DHB 25 mg/mL	1:1 MeOH:Water + 0.2% TFA	1	240
Pretomanid	Pa	Ph-CCA-NH2 5 mg/mL	3:1 ACN:Water	tbd	tbd
Moxifloxacin	M	HCA 7 mg/mL	3:1 EtOH:Water + 0.2% TFA	2	100
Rifapentin	Rpt	tbd	tbd	tbd	tbd

nd: **not detected**; tbd: **to be determined**; HCA: **Alpha-cyano-4-hydroxycinnamic acid**; Ph-CCA-NH2: **4-Phenyl-alpha-cyanocinnamic Acid Amide**; DHB: 2,5-dihydroxybenzoic acid; EtOH: **Ethanol**; TFA: **Trifluoroacetic Acid**; ACN: **Acetonitrile**; MeOH: **Methanol**

Not surprisingly, LLOQs appear to be high when compared with measurements that can be obtained by standard LC-MS/MS methods applied to tissue homogenates (see Figure in the *in vivo* studies). However, this is not a matter of concern provided the concentrations measurable fit with compound levels achievable in *in vivo* efficacy experiments. MALDI MSI is intended not to replace standard PK methodologies but to complement them providing information about micro-distribution in the tissues.

In general terms, there's a reasonable agreement between measurements in IBT and GSK with greater discrepancies only in the case of Moxifloxacin. The difference could be explained by a number of technical reasons also including differences in the sample processing, instruments and the analytical software. Overall, it has also been observed that linear range is wider in IBT (higher ULOQs) than in GSK. A dedicated sub-team will explore further harmonization in software platforms that is expected to reduce the differences. Progress will be reported in due course.

iv. *In vivo* Proof of Concept

In order to determine if the analytical performance achievable using *in vivo* samples was comparable to that obtained in *in vitro* test (method performance in real conditions assay) *in vivo* proof of concept (PoC) studies were carried out using Moxifloxacin and Ethambutol administered to non-infected mice.

Both compounds were administered by oral route to C57BL/6 mice at the same dose, 100 mg/kg. Lung samples were obtained at 0.75, 6 and 24h post-dose and split in two parts. One part was homogenated to allow compound quantification by standard LC-MS/MS quantification. Second part was processed for MALDI-MSI analysis. Time point of 0.75 h post dose was selected as closer to Tmax based on previous mouse PK studies performed with these compounds (data not shown).

MALDI MSI was performed for both compounds at IBT and GSK. Compound quantification in lung homogenates was performed only in GSK. IBT results for Moxifloxacin and Ethambutol results are presented below in Figure 6 and Figure 7, respectively.

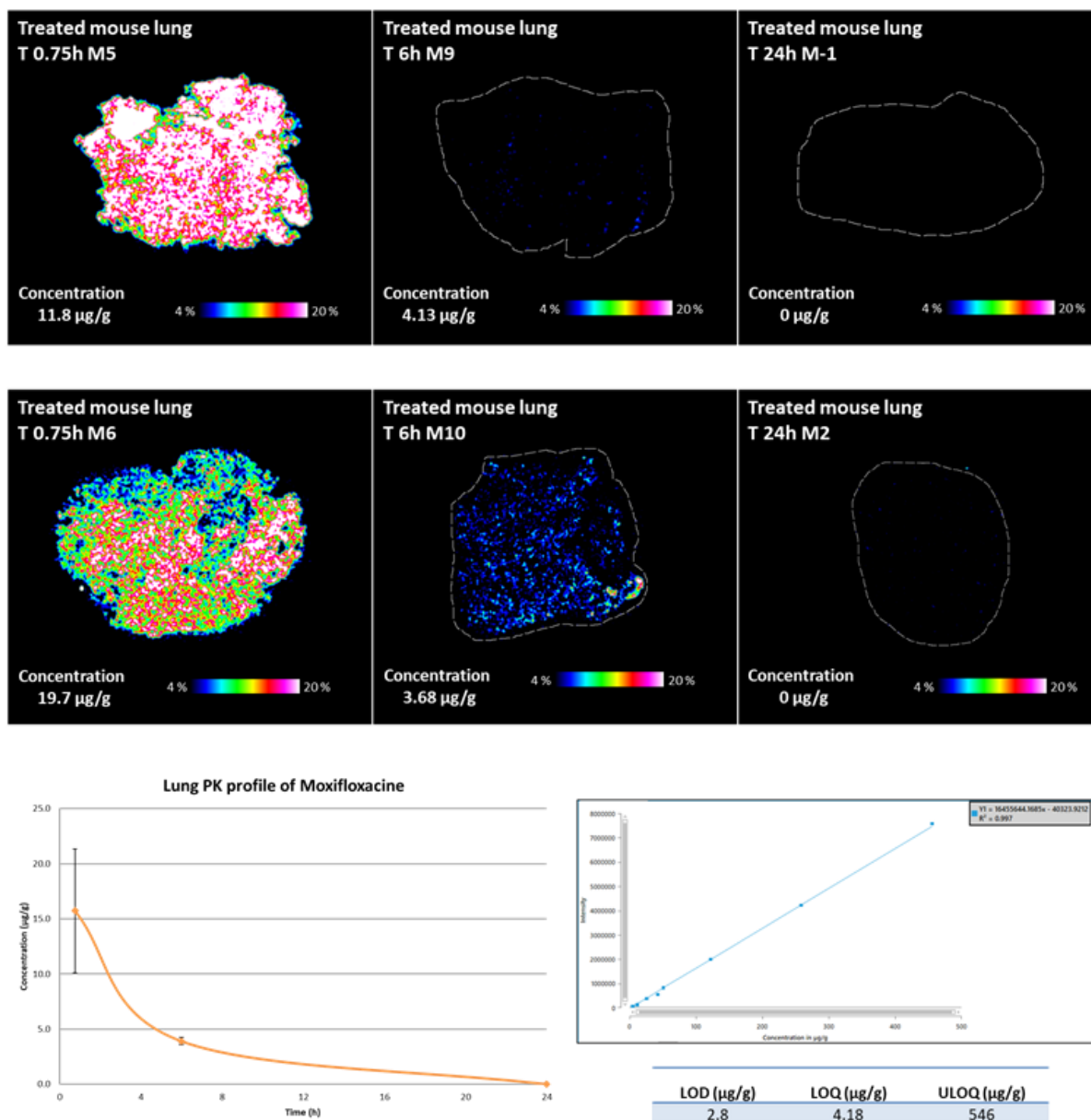


Figure 6: Moxifloxacin PK in lung studied by MALDI MSI (IBT)

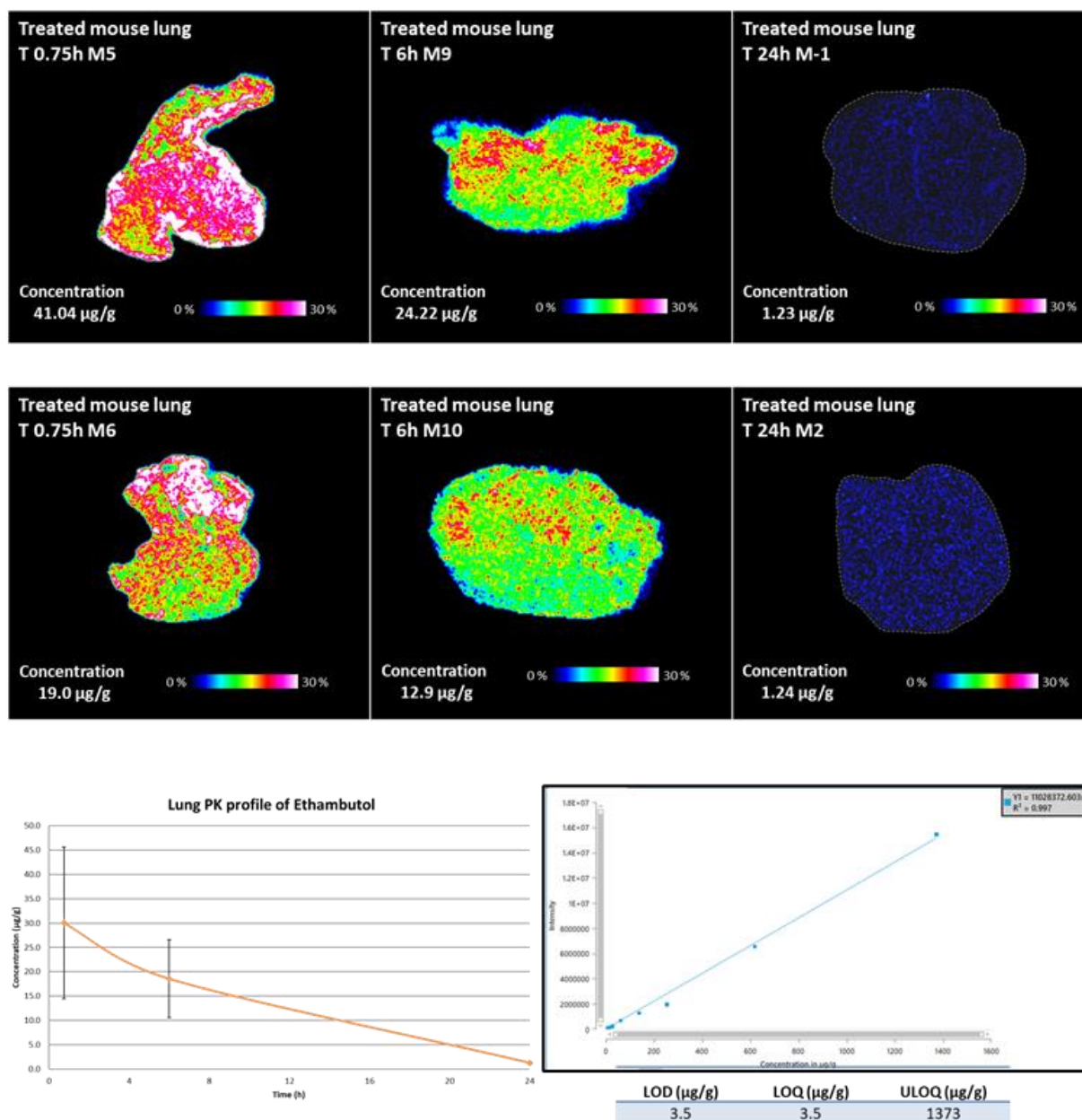
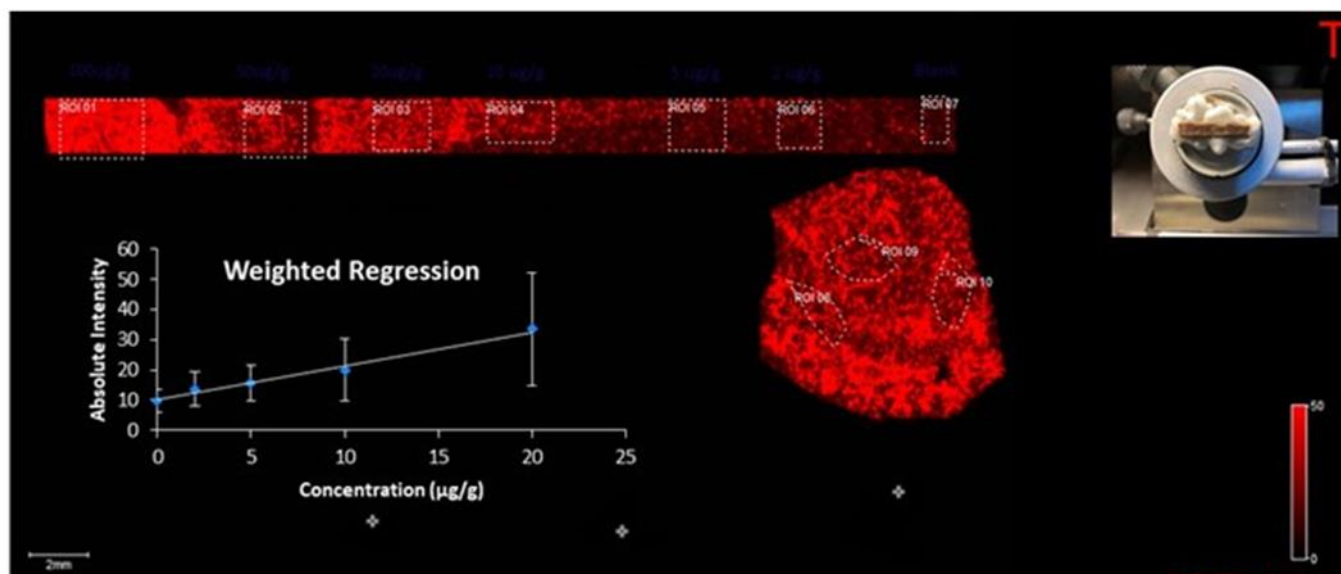


Figure 7: Ethambutol PK in lung studied by MALDI MSI (IBT)

During this *in vivo* PoC, MALDI MSI allowed to determine the lung exposure of moxifloxacin and ethambutol with good correlation with LC-MS/MS analysis of lung homogenates (not shown). For the future, deeper analysis at the histological region level would be interesting by correlating MSI with H&E staining's, especially in infected animals at the lesion level.

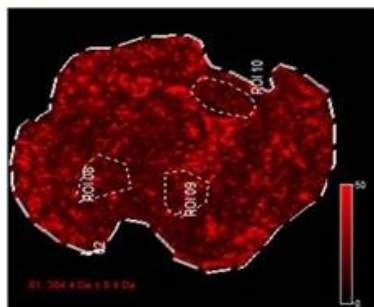
Similarly, lung samples treated with control compounds have been analysed by MALDI MSI at GSK Tres Cantos. Preliminary results of moxifloxacin have shown in Figure 8.



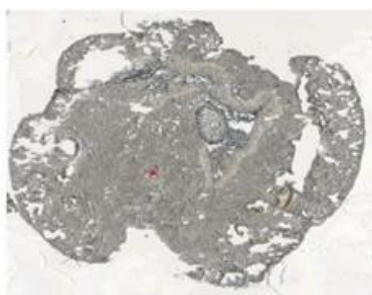
LLQ = 2µg/g

Quantification by MIMETIC standard curve

Samples T 0.75h M7-5



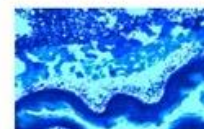
Maldi Image



Optical Image



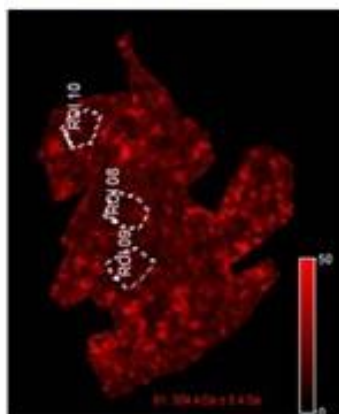
H&E staining Image



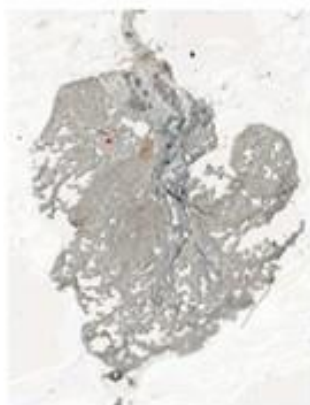
	Mimetic model (MALDI)	Homogenate (LCMS)
Concentration	14.5 ± 3.8 µg/g *	12.7 µg/g

* Concentration of M. tuberculosis in the sample, as determined by LC-MS/MS

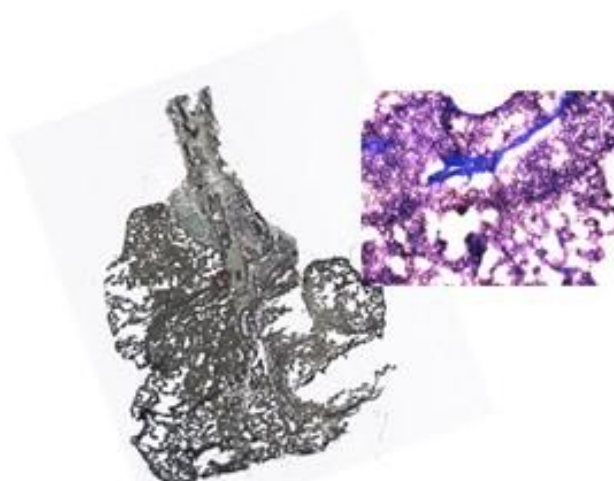
Samples T 6h M11-5



Maldi image



Optical image



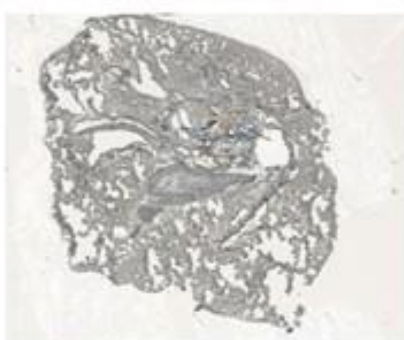
H&E staining image

	Mimetic model (MALDI)	Homogenate (LCMS)
Concentration	$3.5 \pm 2.2 \mu\text{g/g}^*$	$4.6 \mu\text{g/g}$

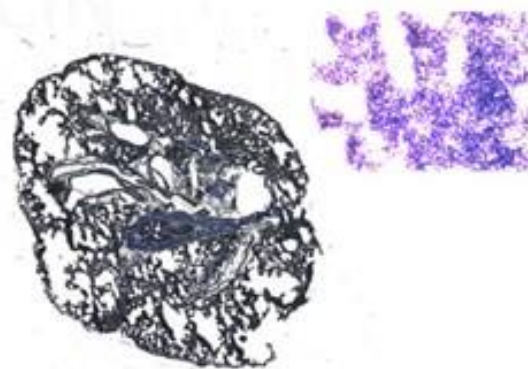
Samples T 24h M3-18



Maldi image



Optical image



H&E staining image

	Mimetic model (MALDI)	Homogenate (LCMS)
Concentration	<LLQ	$0.1 \mu\text{g/g}$

Figure 8: Moxifloxacin PK in lung studied by MALDI MSI (GSK)

Preliminary data suggest a very good correlation with parallel quantification in lung homogenates by LC-MS/MS. Results are also consistent with those obtained at IBT.

8. Summary and current status

Imaging techniques applied to living subjects, organs and tissues, either in vivo or ex vivo, and specifically MALDI MSI, can significantly impact on the development of new drugs in early phases of Drug Discovery (Figure 9) supporting big number of key studies providing information on Safety, Efficacy and Pharmacokinetics of new drugs. In addition, MALDI MSI also will be key on translational PK/PD research provided relevant knowledge of compound levels at target through micro-distribution in necrotic lesions studies (Figure 10).

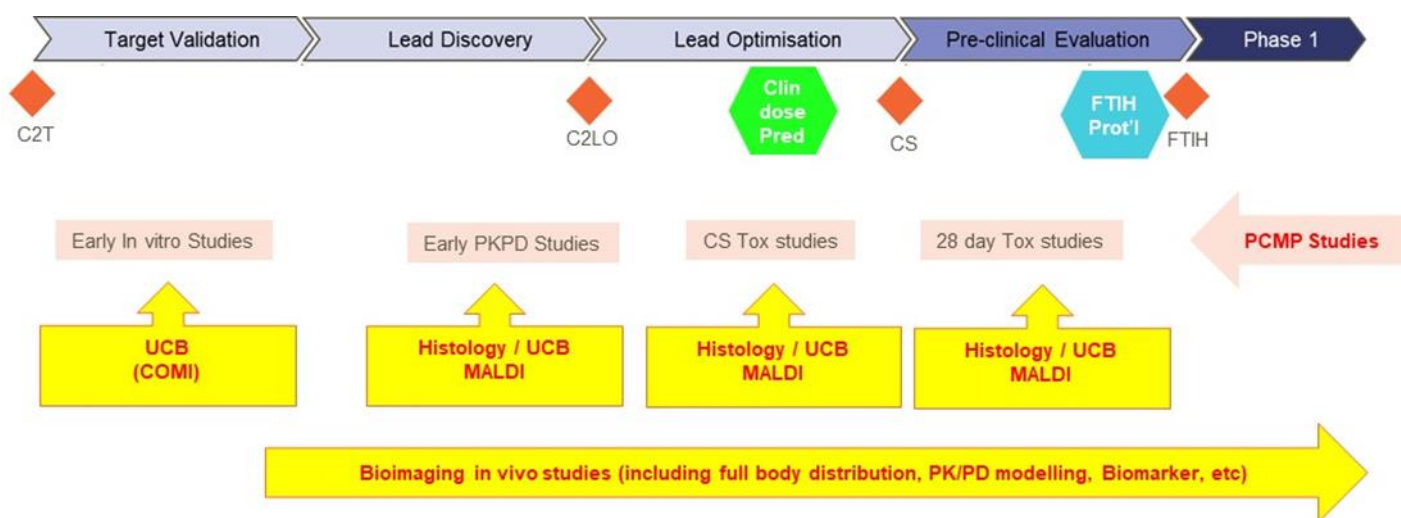


Figure 9: MALDI MSI in early development of new drugs

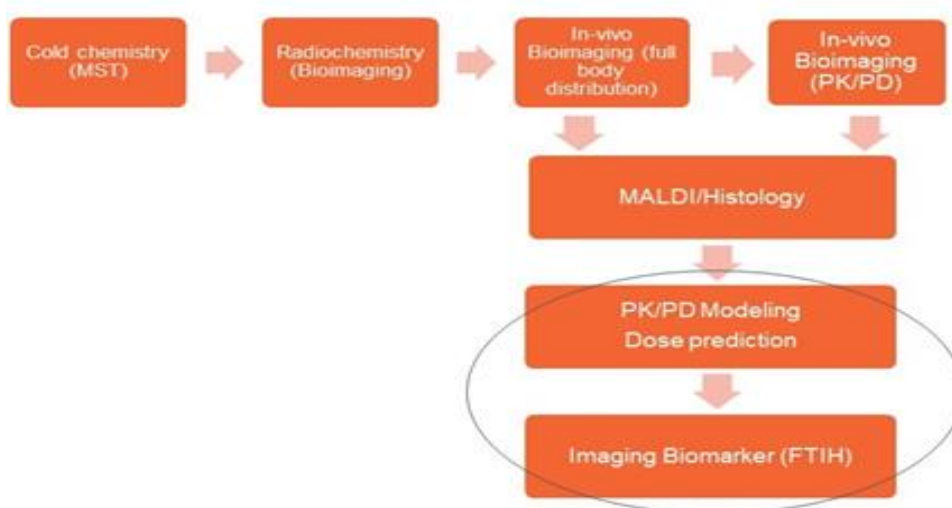


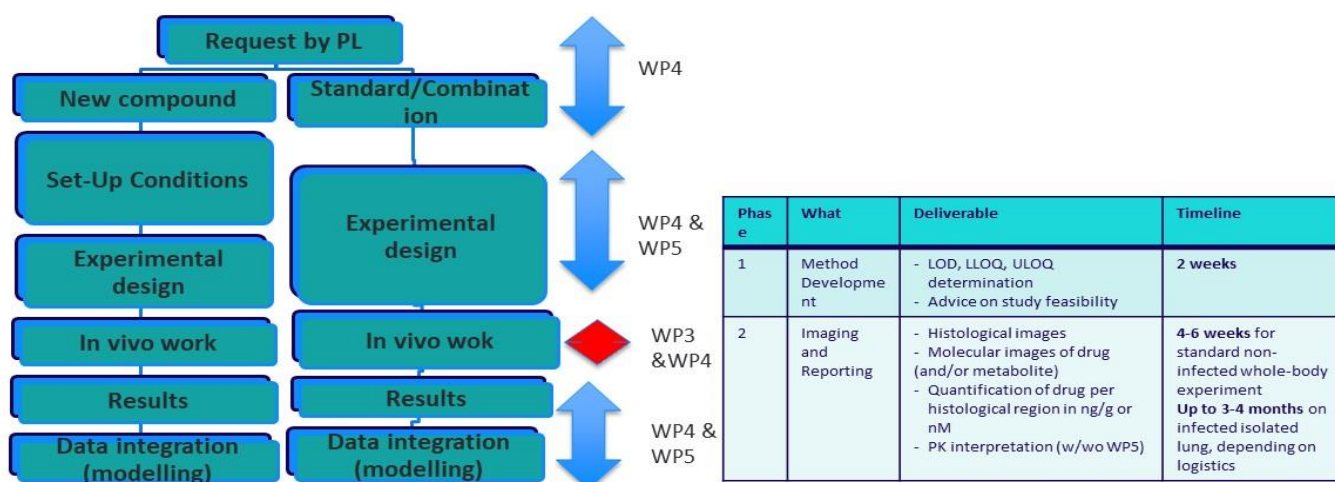
Figure 10: MALDI MSI in translational research

MALDI-MSI platform in ERA4TB require to work with samples infected with Mtb. As it is well known, Mtb is a Biosafety-Level 3 microorganism (BSL-3) so it is only possible to work with Mtb-infected samples either enclosed in BSL-3 facilities or if the sample is previously decontaminated to eliminate viable Mtb bacteria.

Two ERA4TB partners are providing MALDI-MSI capability; GSK at its facilities in Tres Cantos (Spain) and IBT in Loos, France. Each partner is applying a different approach to work safely with Mtb infected samples. GSK has designated a dedicated area in its BSL-3 containment facilities where MALDI-MSI analyser and enabling equipment is placed. This MALDI suit is fully operative. On its turn, IBT has been testing a number of sterilization approaches for samples able to kill Mtb bacteria thus preserving histological integrity and not modifying compound's concentration or distribution.

Although there's still need of further improvement and consolidation, at present the consortium is able to attend MALDI-MSI work requests required for pipeline progression coming from ERA4TB partners.

An initial Workflow to request specific MALDI MSI assays in ERA4TB consortium has been agreed and is presented Figure 11. It is expected that this workflow will be fine-tuned based on ERA4TB pipeline progression and partners requests. It is considered always to undergo a bespoke design of the experiments depending of the specific questions to be addressed. Timelines will depend of the final experimental designs and slot availability *in vivo*.



WP4: WP4: Imaging; **WP3:** *In vivo* studies; **WP5:** Modelling

Figure 11: MALDI MSI Study Workflow including interaction with other ERA4TB Work Packages (WP)

9. Annexes

i. Annex (I): Set up of analytical methods at IBT. Extended data

For each compound and analytical protocol, the parameters determined have been Limit of Detection (LOD), Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ)

Isoniazid (H)

Despite our attempts, no suitable method has been developed so far. Considering the key place of this drug in the therapies, further efforts will be considered in 2022.

Rifampicin (R)

A first method with 9-Aminoacridine (9AA) has been developed. It led to a moderate sensitivity with a LOD of 23.4 µg/g.

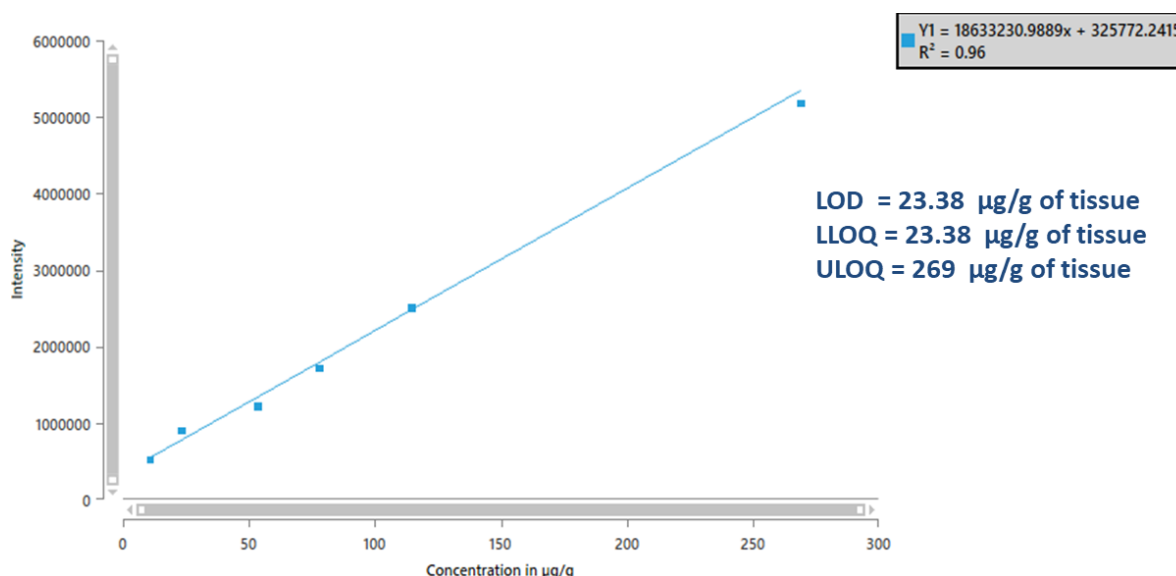


Figure 12: Calibration curve of Rifampicin with 9-AA

Further developments with para-nitroaniline (pNA) improved the sensitivity with a LOD of 4.5 µg/g. Unfortunately, this matrix tends to sublime and appears to be unsuitable for long imaging runs as can be required by high spatial resolution imaging experiments. If qualitative information on whole-body mouse is of interest, this matrix could still be a sensitive option.

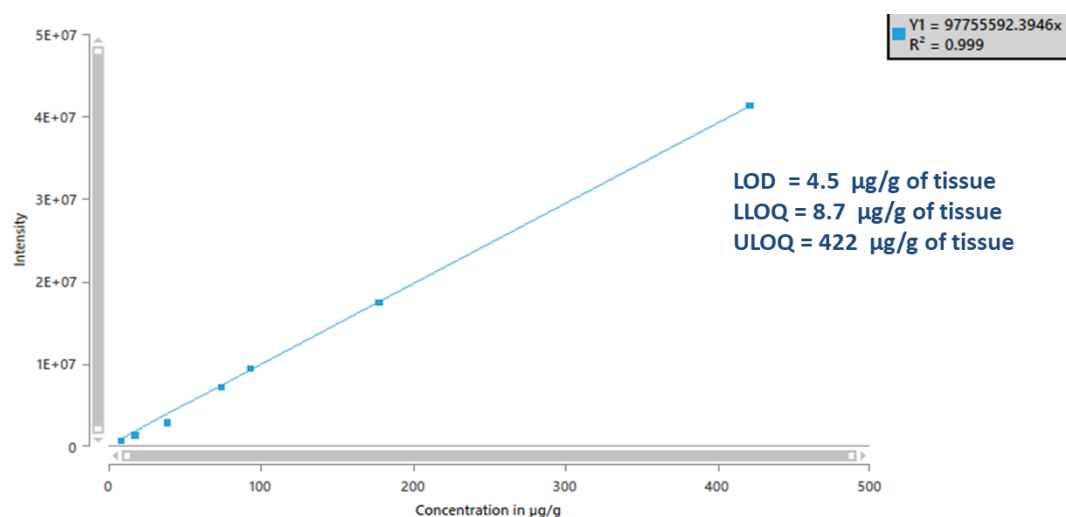


Figure 13: Calibration curve of Rifampicin with pNA

Pyrazinamide (Z)

Despite our attempts, no suitable method has been developed so far since the LOD achieved was 158 µg/g which is well above our MSI standards. Considering the key place of this drug in the therapies, further efforts will be considered in 2022.

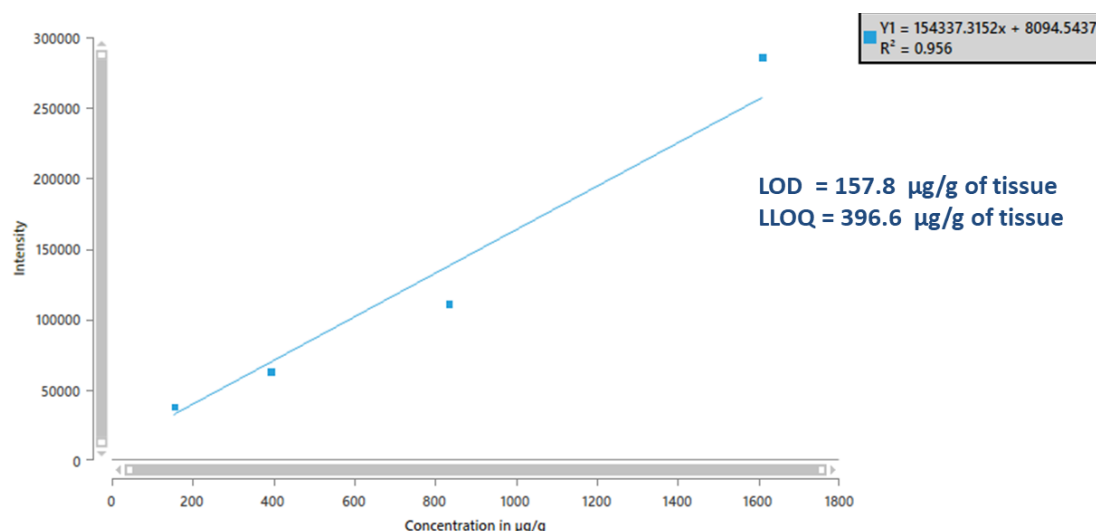


Figure 14: Calibration curve of Pyrazinamide with DHB

Ethambutol (E)

Ethambutol's performance was close to the normal range of sensitivity of MSI, in the high hundreds of ng/g range.

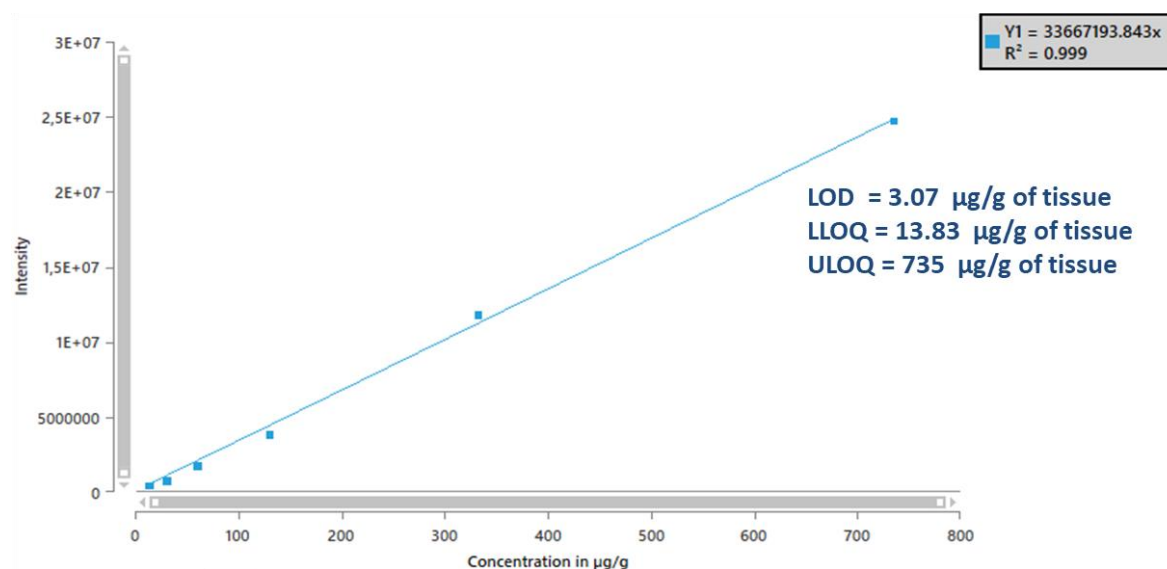


Figure 15: Calibration curve of Ethambutol with DHB

Bedaquiline (B)

Bedaquiline's performance was close to the normal range of sensitivity of MSI, in the high hundreds of ng/g range.

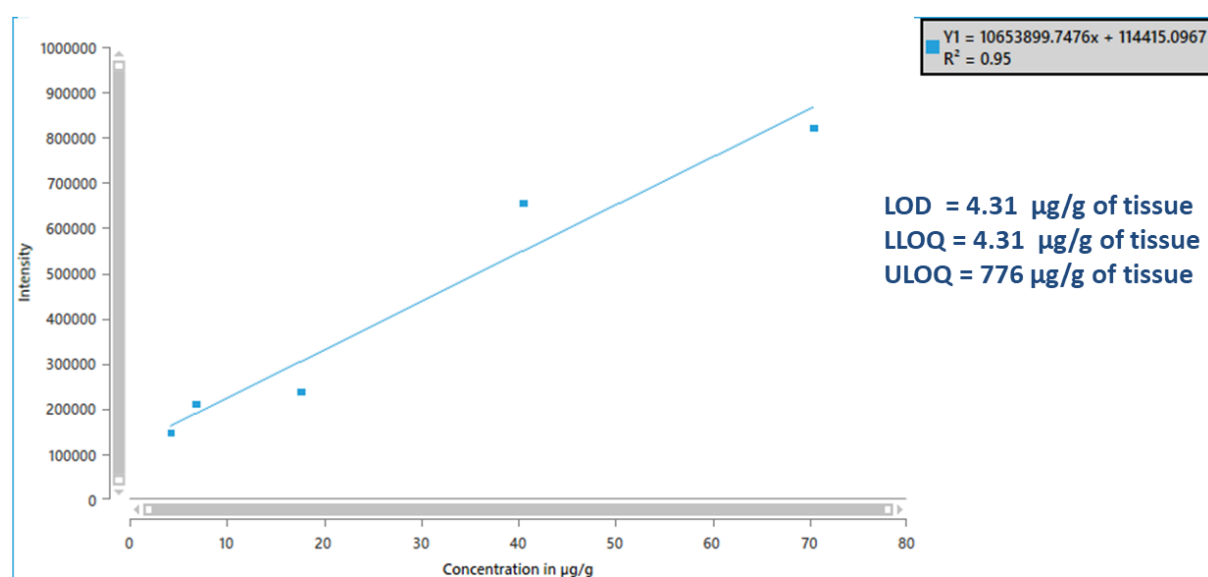


Figure16: Calibration curve of Bedaquiline with DHB (low concentration range)

Pretomanid (Pa)

Pretomanid had a moderate sensitivity with a LOD of 11.8 µg/g.

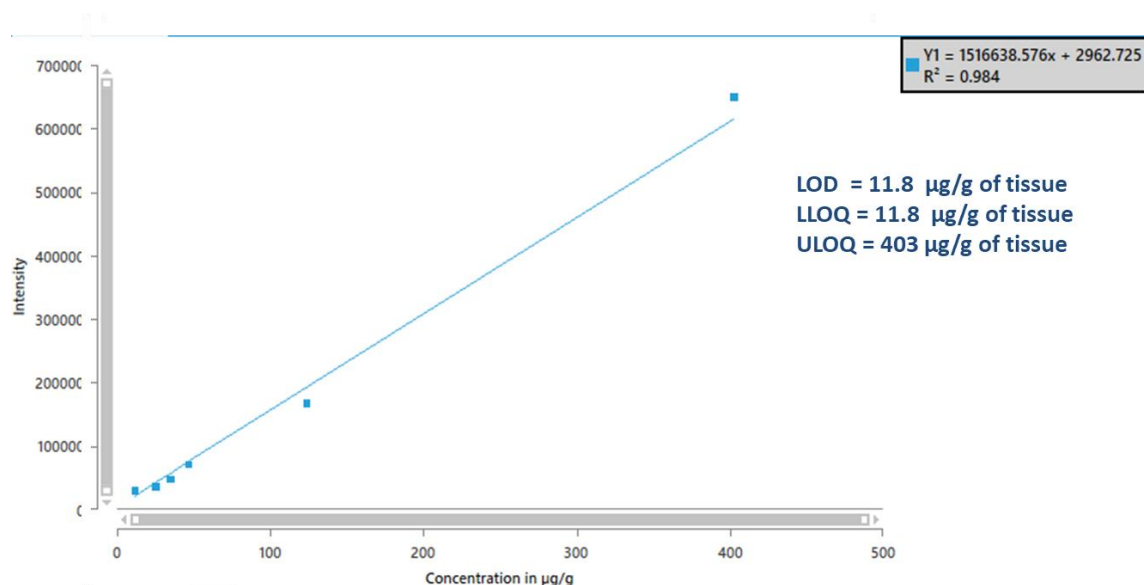


Figure 17: Calibration curve of Pretomanid with DHB

Moxifloxacin (M)

Moxifloxacin performance was good, with a LOD at 3.24 µg/g.

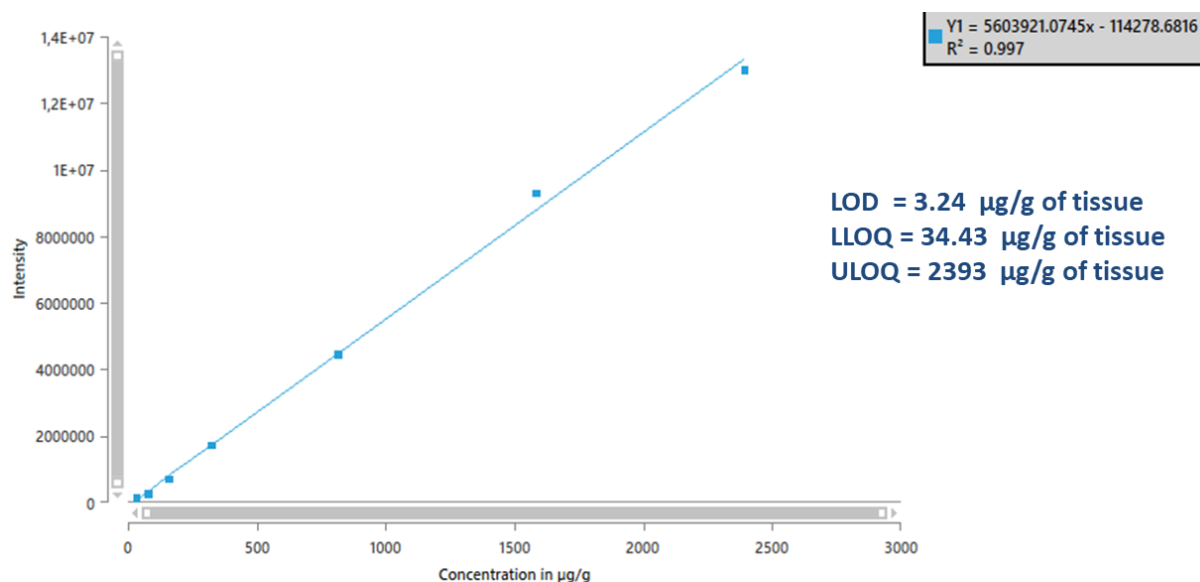


Figure 18: Calibration curve of Moxifloxacin with DHB

Rifapentine (Rpt)

Although Rifapentine share some common structural features with rifampicin, it gave a much better analytical performance with 9AA with a LOD of 3.56 µg/g.

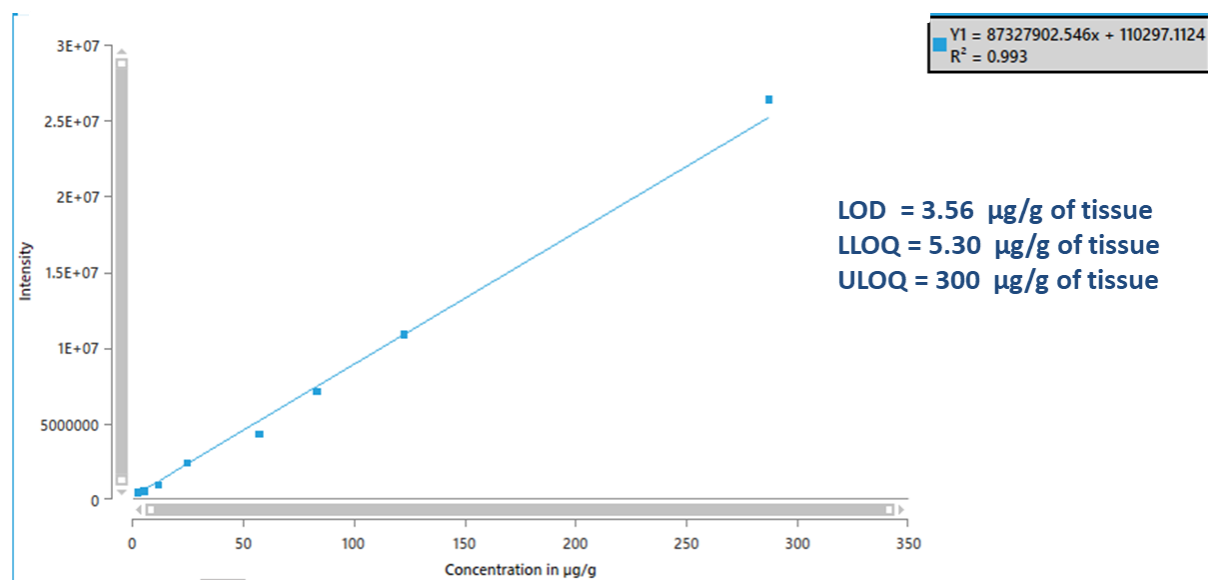


Figure 19: Calibration curve of Rifapentine with 9AA