



RapidFire for High Throughput Screening in early Drug Discovery

Fast but do we stay on track?

Markus Trunzer



ADME Profiling in Basel

High analytical burden

~8'000 compounds per year by HT-Sol and HT-Perm



7 injections per compound for HT-Perm

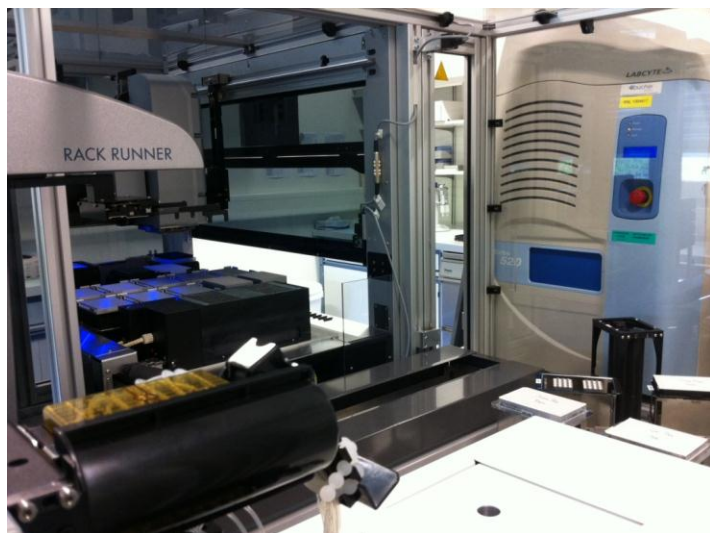
15 injections per compound for HT-Sol

~176'000 LC-injections per year



2 min per injection

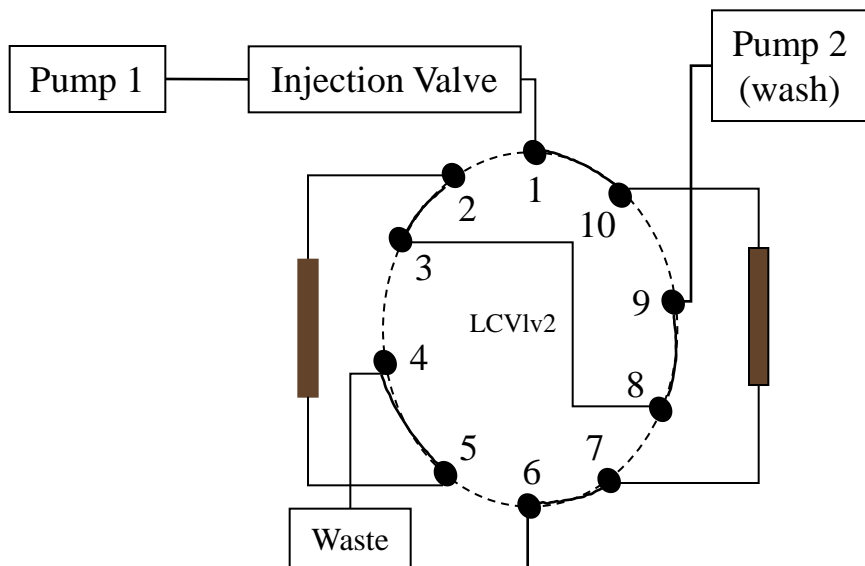
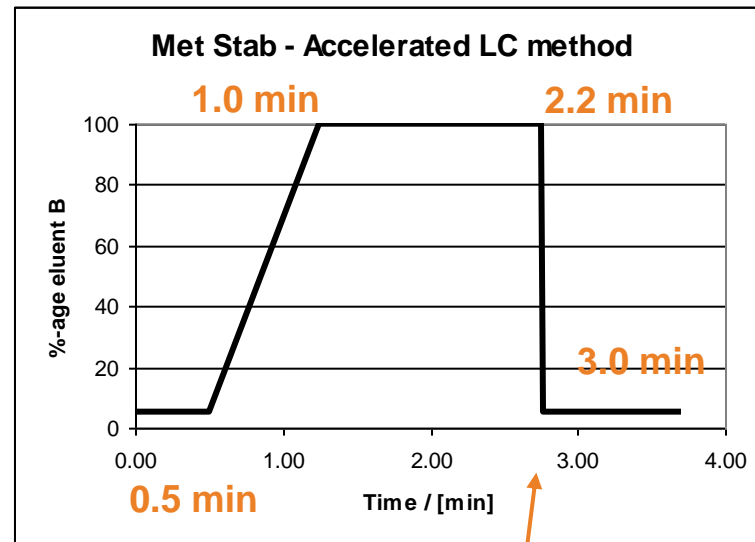
~5'867 hrs per year (1 year = 8'760 hrs)



Generic Analytical Equipment with 5 LC-MS/MS

Short LC method and dual column used for all assays

| | |
|-------------|---|
| Solvent A | Water with 0.1% HCOOH |
| Solvent B | Acetonitrile with 0.1% HCOOC |
| Column | Synergi RP Polar, 2.0 x 50 mm, 4.0 μm |
| Temperature | 50°C |
| Flow rate | 350 μ L/min |



Tuning
Acquisition

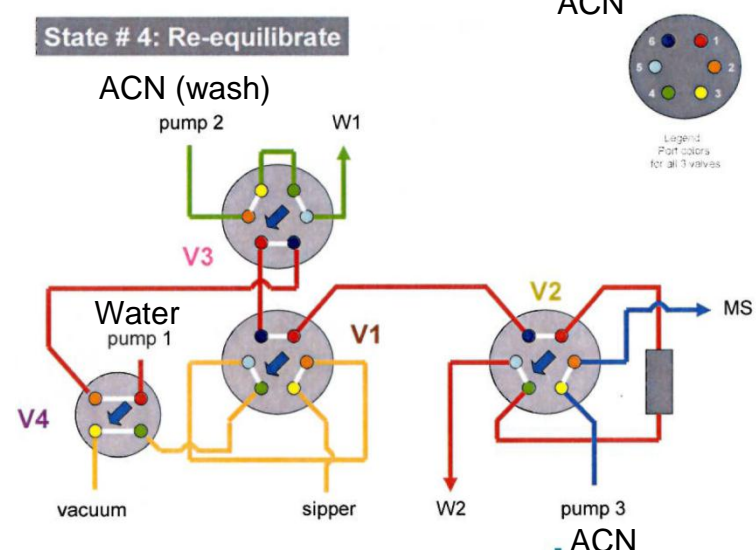
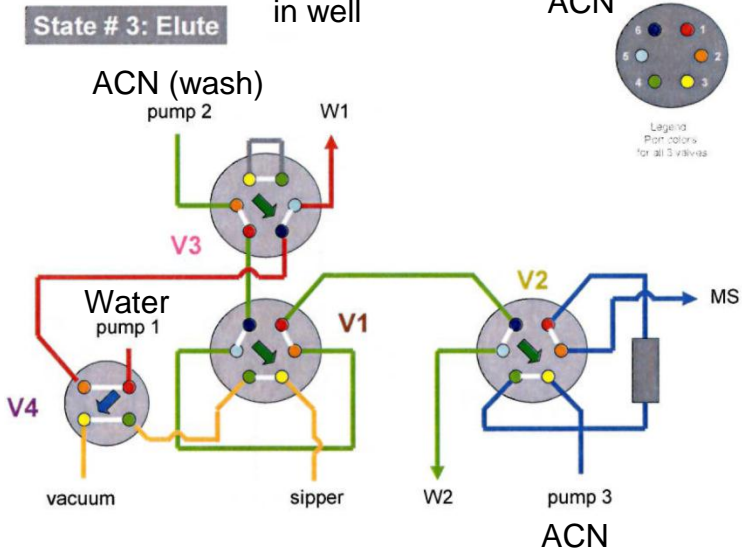
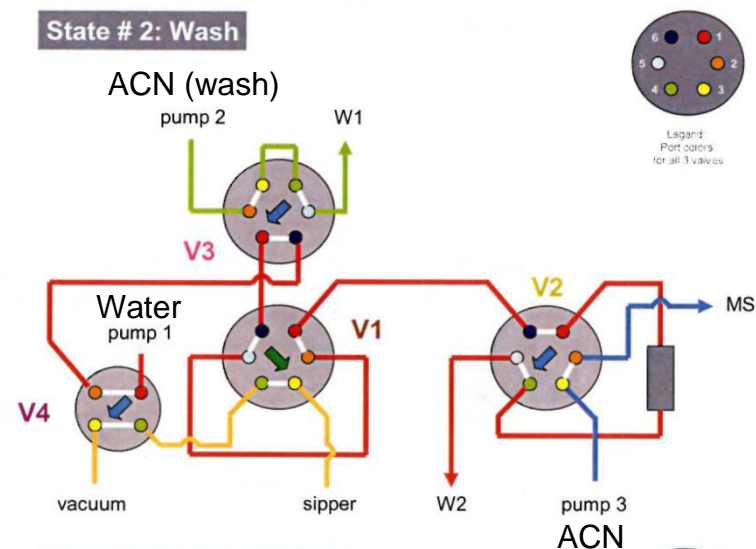
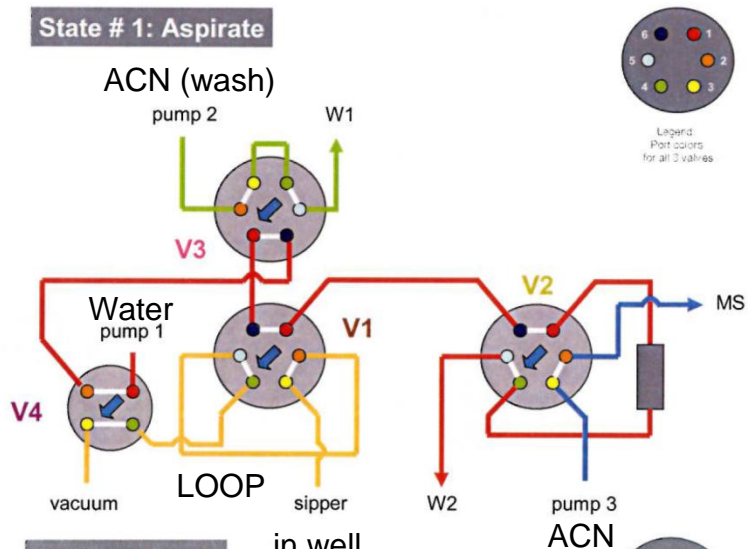


Tuning Database

Column switching

Flow Path of the RapidFire

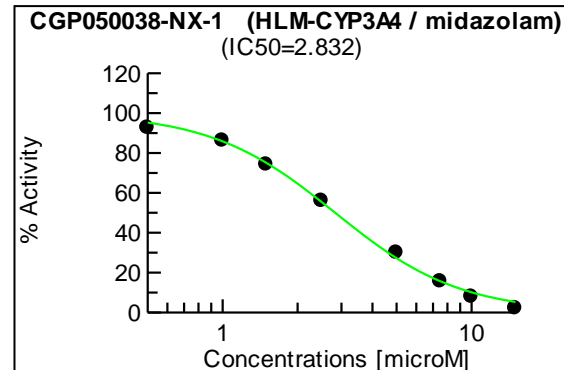
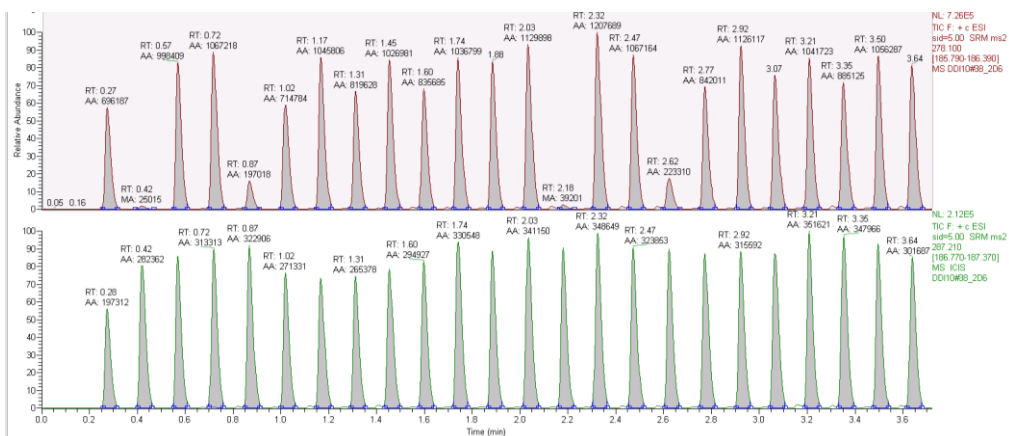
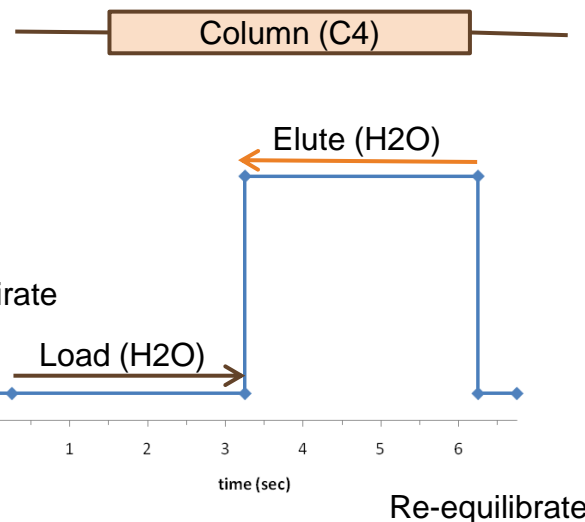
Fast & Automated on-line SPE System



Method RapidFire-TSQ for DDI

Aspirate: **250 ms**
 Load/Wash: **3000 ms**
 Elute: **3000 ms**
 Re-equilibrate: **500 ms**
 Buffer A: 0.1 % formic acid in water
 Buffer B: 0.1 % formic acid in acetonitrile
 Column: C4
 Pump 1: 1.5 ml/min (water to aspirate/wash)
 Pump 2: 1.25 ml/min (acetonitrile wash loop)
 Pump 3: 1 mL/min (acetonitrile for elution)

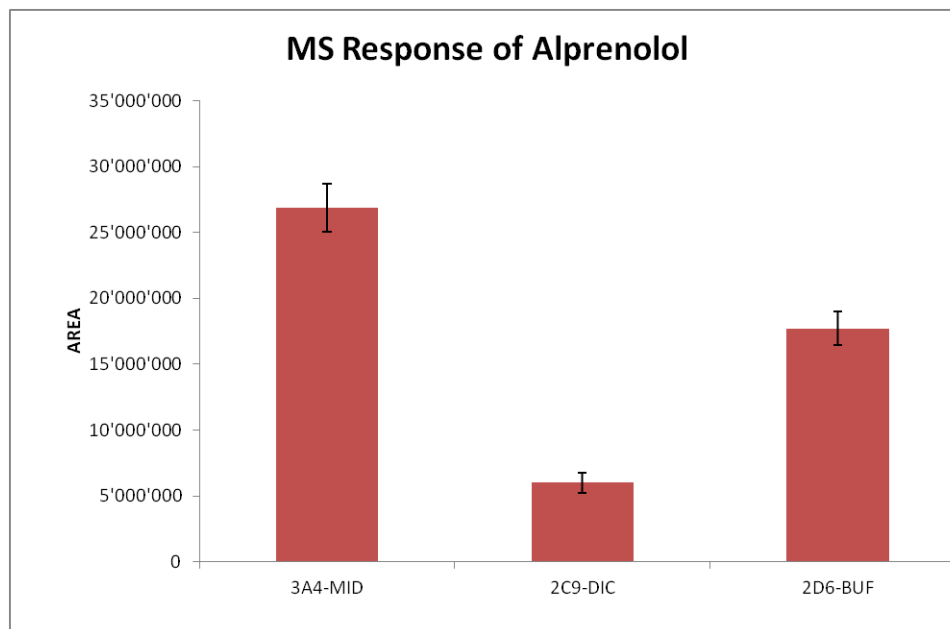
Cycle time: **8.6 s (measured)**
 Run-time: **54 min for 384 injections**



Issues and Solutions

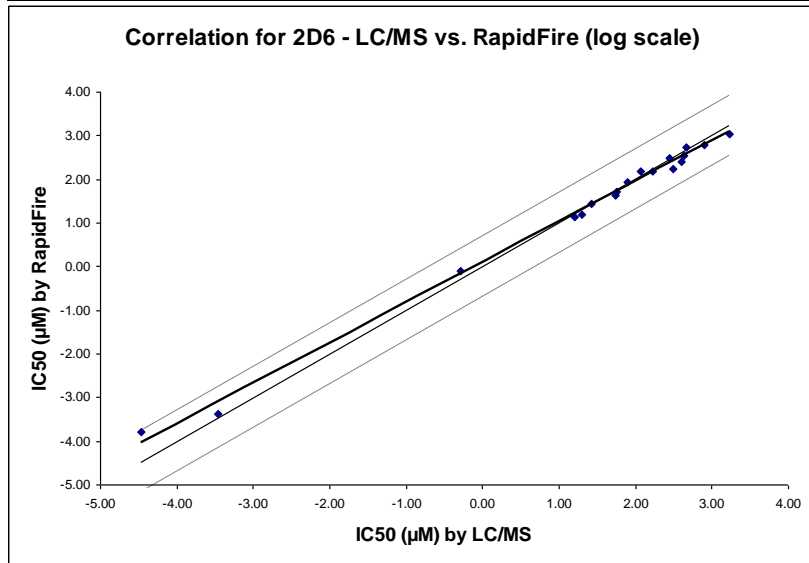
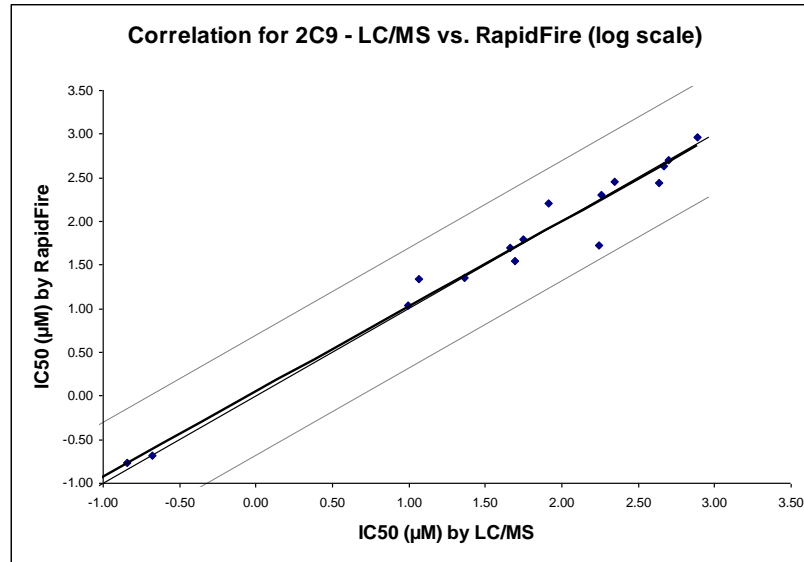
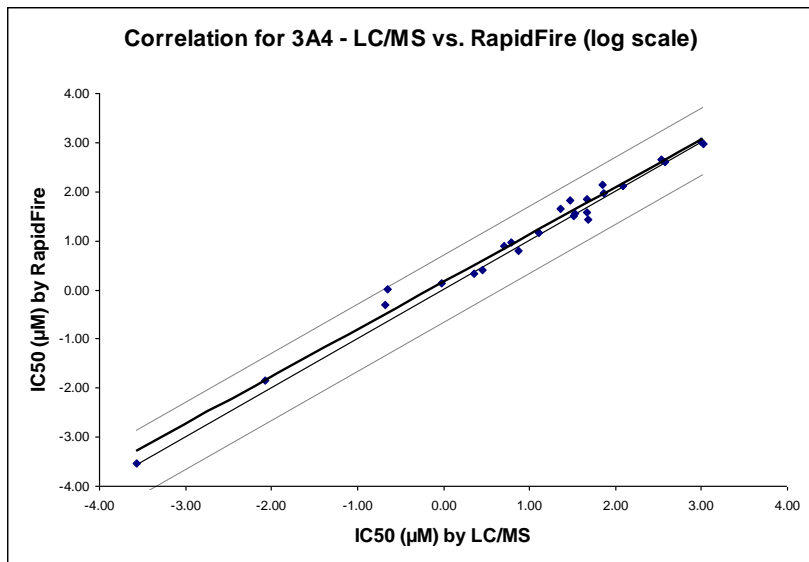
Lower signals

- Higher flow rate = lower response
 - Change of the interface (H-ESI)
 - No pooling of incubations
 - Higher concentration of internal standards
- No separation = ion suppression effects
 - Change of sample work-up



Correlation of IC50s by LC/MS and RapidFire

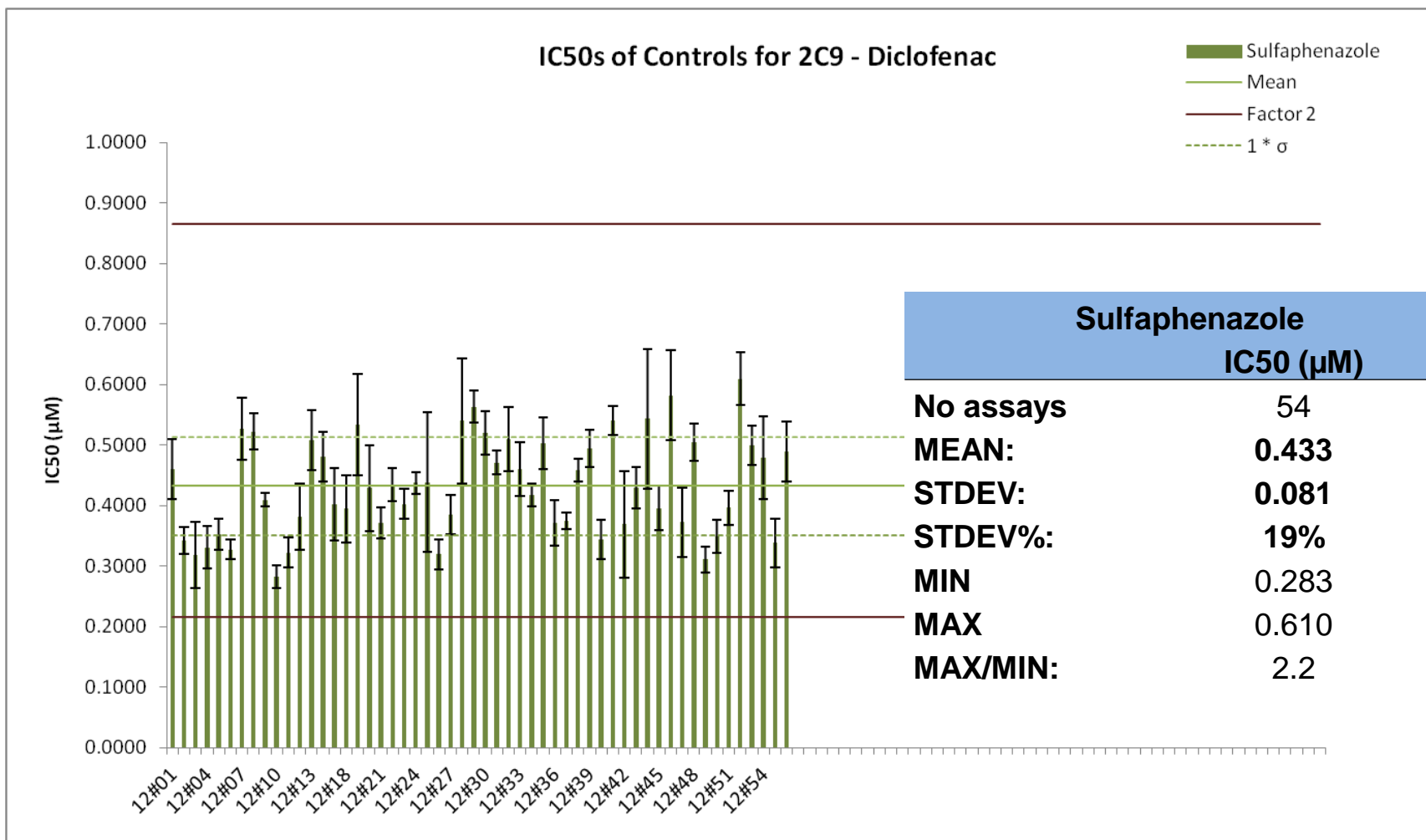
Good correlation – no issues



| Iso-Enzyme | Correlation: | log-scale |
|------------|----------------|-----------|
| 3A4 | slope | 0.964 |
| | intercept | 0.146 |
| | R ² | 0.985 |
| 2C9 | slope | 0.974 |
| | intercept | 0.044 |
| | R ² | 0.980 |
| 2D6 | slope | 0.926 |
| | intercept | 0.104 |
| | R ² | 0.996 |

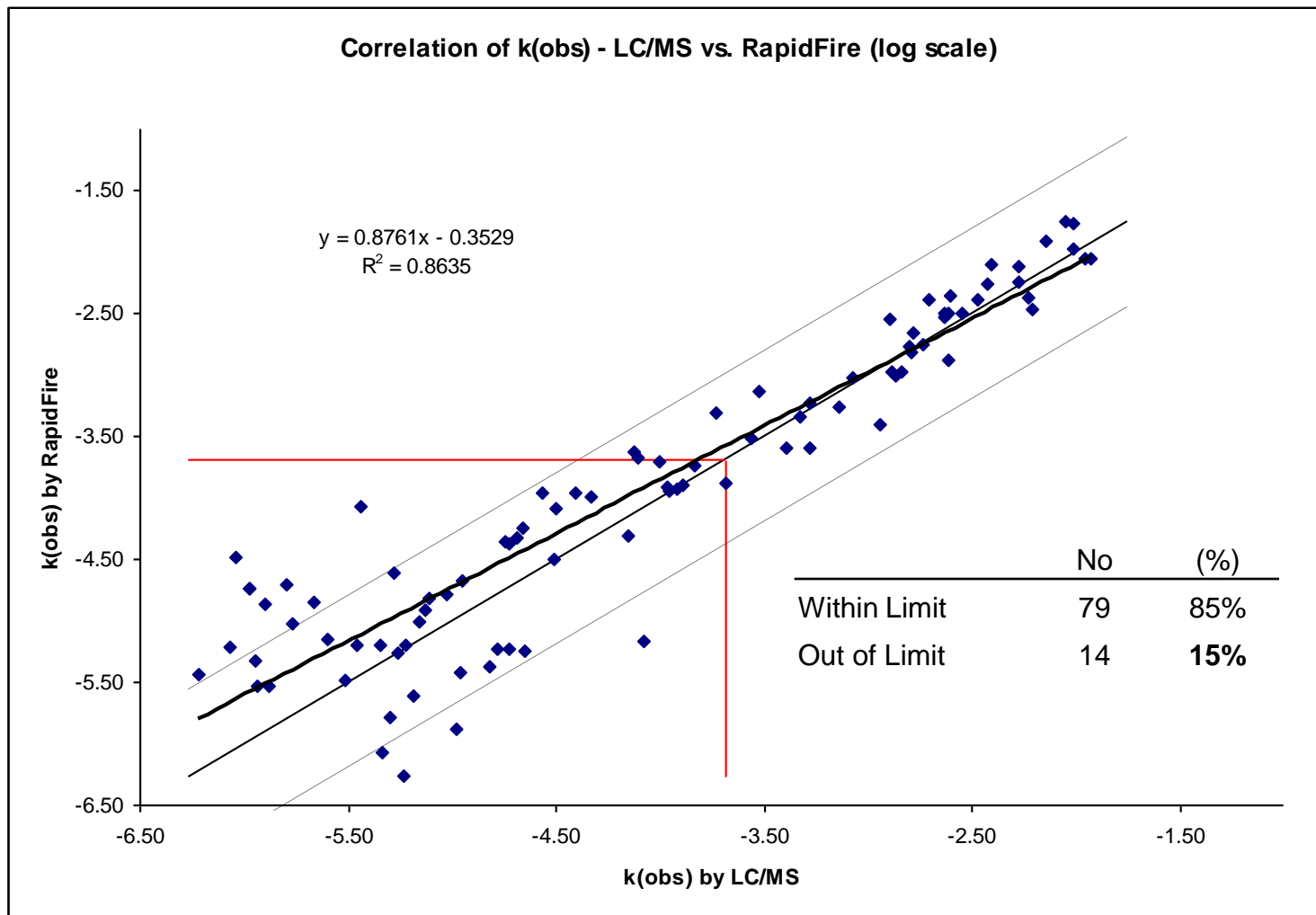
Only

Reliable determination of IC50s for Sulfaphenazole *OH-Diclofenac for 2C9 - the worst case for MS-analysis*



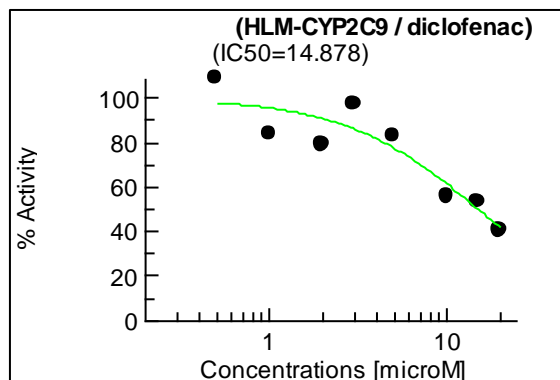
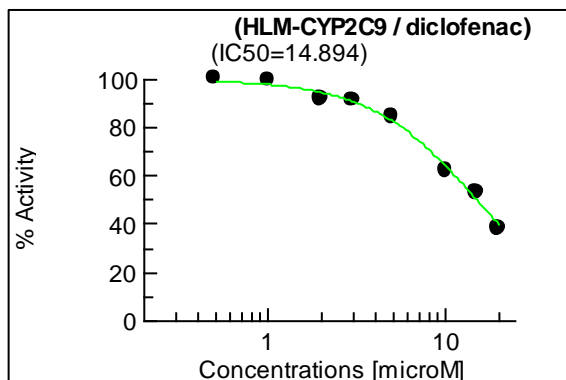
Correlation Time-Dependent Inhibition

LC-MS/MS compared to Rapid Fire



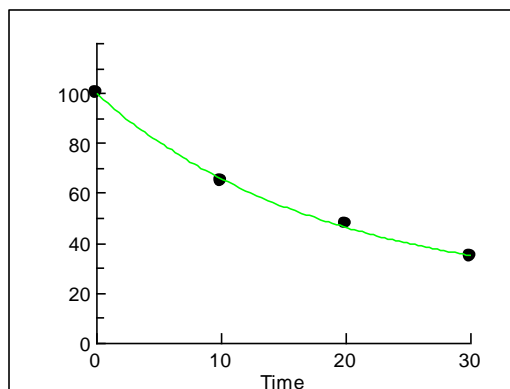
Missing Precision

Averaging effect with IC50-determination but not with rate

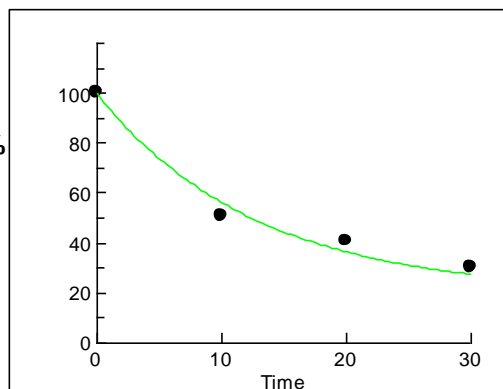


| | LC/MS | RapidFire |
|------|-------|-----------|
| IC50 | 14.9 | 14.9 |
| SE | 0.6 | 3.0 |
| %SE | 4% | 20% |

Averaging effect for the determination of IC50 values



delta
max 14%



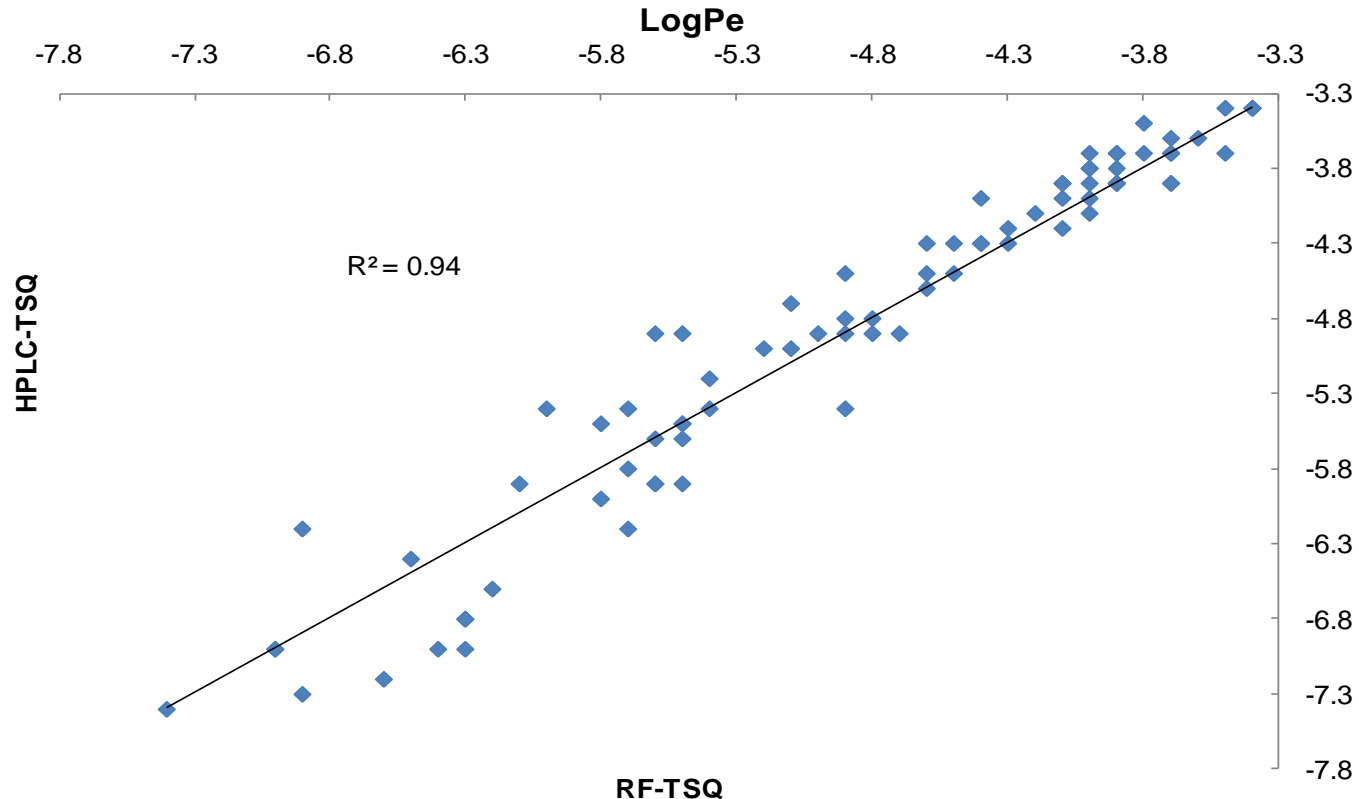
| | LC/MS | RapidFire |
|-----------|--------|-----------|
| k(obs) | 0.055 | 0.079 |
| SEk(obs) | 0.0012 | 0.0080 |
| %SEk(obs) | 2% | 10% |

43%

Small deviations in single points (14%) lead to strong discrepancies in the result (43%)

Permeability with RF-TSQ

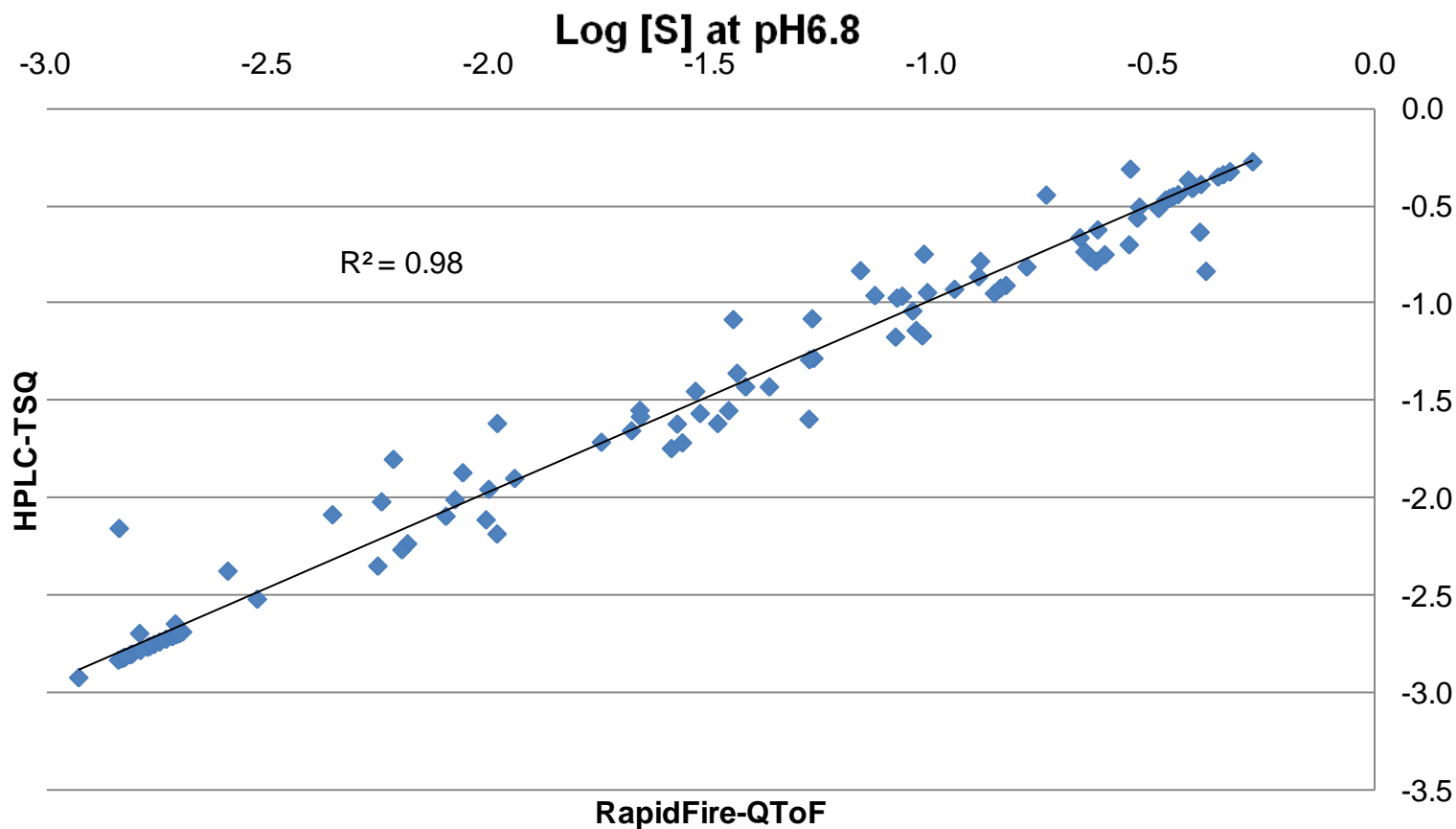
Analysis for the test article itself



- Possible, but requires a complicated work-flow
 - Synchronizing RapidFire with TSQ
 - The required frequent stop/start of the TSQ slows down the system

Solubility with RF-Q-ToF

Analysis for the test article with high-resolution and exact mass



The MetStab Assay

Low concentrations and high amount of impurities

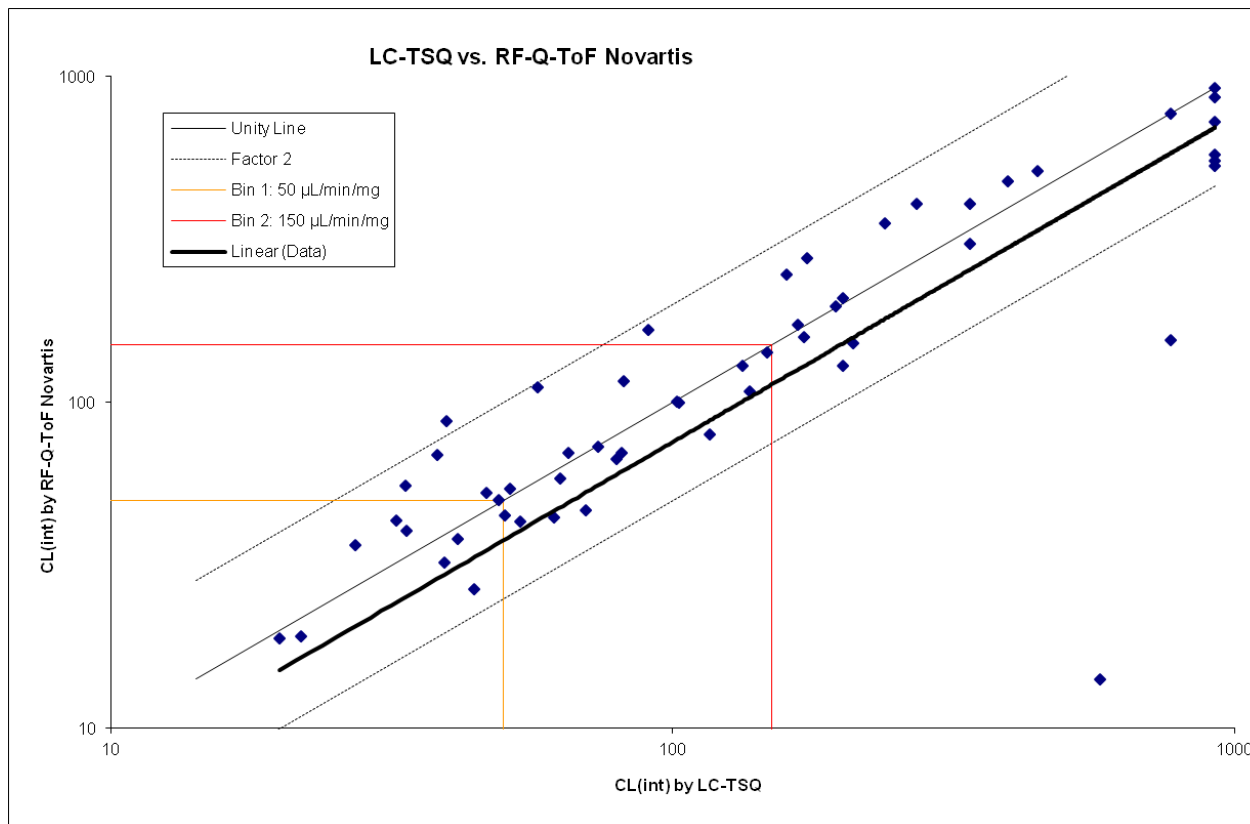
- Incubations with 1 μM substrate and 0.5 mg/mL protein of HLM
- Stop of incubations with 3 times ACN
- Organic content (75%) is reduced by evaporation in a speed-vac (not to dryness) and re-suspended in water

| | Conc. (μM) | Injected amount (pmol) |
|------|-------------------------|------------------------|
| 100% | 0.25 | 2.5 |
| 10% | 0.025 | 0.25 |
| 1% | 0.0025 | 0.025 (= 8 pg*) |

* Assuming a molecular weight of 300 g/mol

LC-MS/MS vs. RF-Q-ToF 6530 (Novartis)

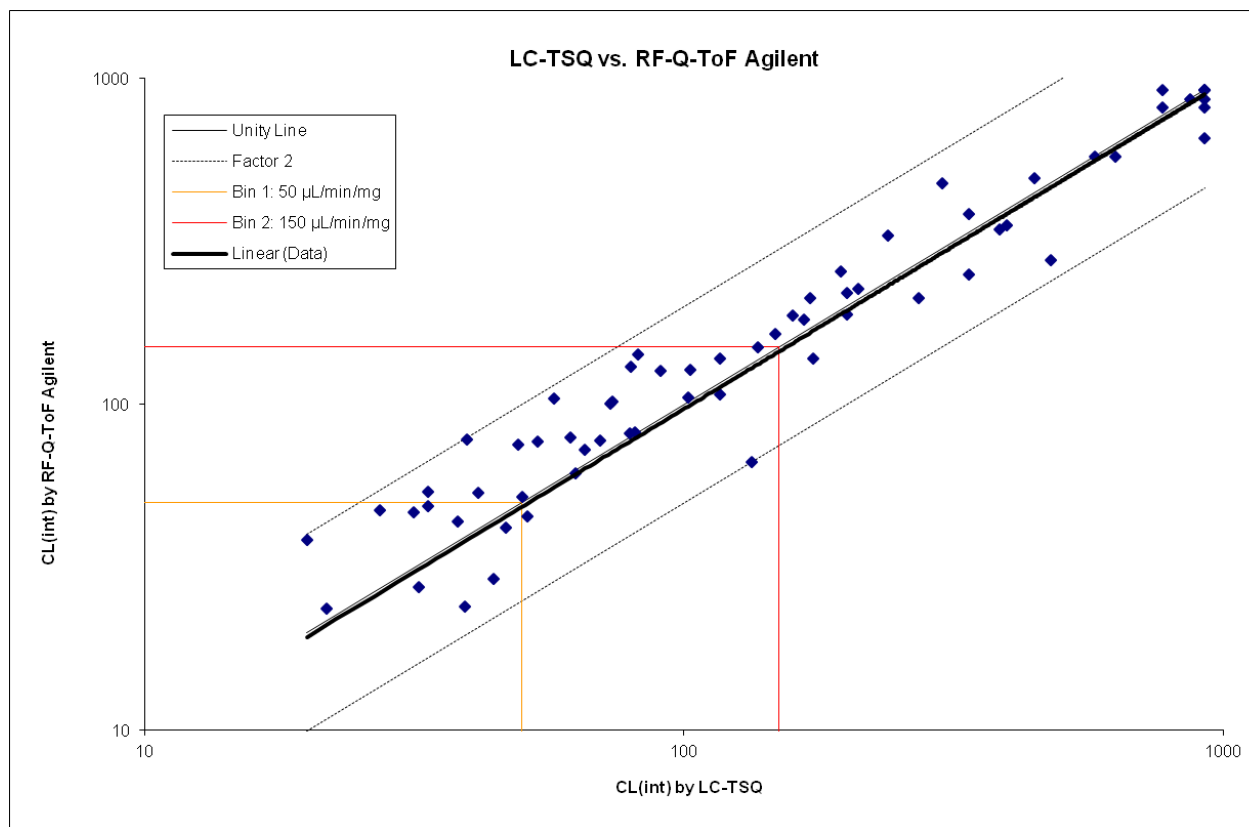
Large amount of missing compounds with RF-Q-ToF



- **21** missing compounds with the RF-Q-ToF whereas only **8** are missing by LC-MS/MS

LC-MS/MS vs. RF-Q-ToF 6550 (Agilent)

Analyzed by Moritz Wagner in Waldbronn



- Similar amount of compounds **7** missing with the RF-Q-ToF 6550 compared to **8** compounds missing by LC-MS/MS

The numbers

| Per 100 Compounds | CYP Inhibition | | | CL _{int} | |
|-------------------|----------------|--------|--------|-------------------|--------|
| | LC-TSQ | RF-TSQ | RF-ToF | LC-TSQ | RF-ToF |
| Samples | 800 | 2'400 | 2'400 | 133 | 400 |
| LC time/Spl (sec) | 210 | 7 | 7 | 120 | 7 |
| Total LC time (h) | 47 | 5 | 5 | 4 | 1 |
| Target LLOQ (μM) | na | na | na | 0.025 | 0.025 |
| % detected at TL | na | na | na | 95% | 80% |

The numbers

| Per 100 Compounds Samples | Solubility | | | Permeability | |
|-------------------------------|------------|--------|--------|--------------|--------|
| | LC-UV | LC-TSQ | RF-ToF | LC-TSQ | RF-TSQ |
| LC time/Spl (sec) | 1'500 | 500 | 1'500 | 700 | 1'500 |
| Total LC time (h) | 120 | 120 | 7 | 120 | 30 |
| Target LLOQ (μM) | 50 | 17 | 3 | 23 | 13 |
| % detected at TL | 4 | 4 | 4 | 0.025 | 0.025 |
| | 95% | 95% | 95% | 95% | 95% |

Conclusion

Do we stay on track?.....not always but issues can be covered

- DDI: no problem with minor changes, averaging effect helps
- TDI: possible with more data points
- Solubility with RF-Q-ToF – no issues (need to dilute!)
- Metabolic Stability and Permeability with next generation Q-ToF
- What did we get?
 - Machine time
 - Turnaround
 - Flexibility
 - Opportunity to develop and run mechanistic assays for better understanding

Acknowledgement

Novartis discovery ADME:

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Gaëlle Chenal

Alfred Zimmerlin

Agilent:

Moritz Wagner

Carl-Heinz Reiner

Holger Stalz

Maxine Jonas

Back-ups

Method for RapidFire

Default Method for the RapidFire (modified for DDI and TDI)

| | |
|------------------|--------------------------------------|
| Aspirate: | 250 ms |
| Load/Wash: | 3000 ms |
| Elute: | 3000 ms |
| Re-equilibrate: | 500 ms |
| Buffer A: | 0.1 % formic acid in water |
| Buffer B: | 0.1 % formic acid in acetonitrile |
| Pump 1: | 1.5 ml/min (water to aspirate/wash) |
| Pump 2: | 1.25 ml/min (acetonitrile wash loop) |
| Pump 3: | 1 mL/min (acetonitrile for elution) |
| Injection-cycle: | 8.6 s |
| Run-time: | 54 min for 384 injections |

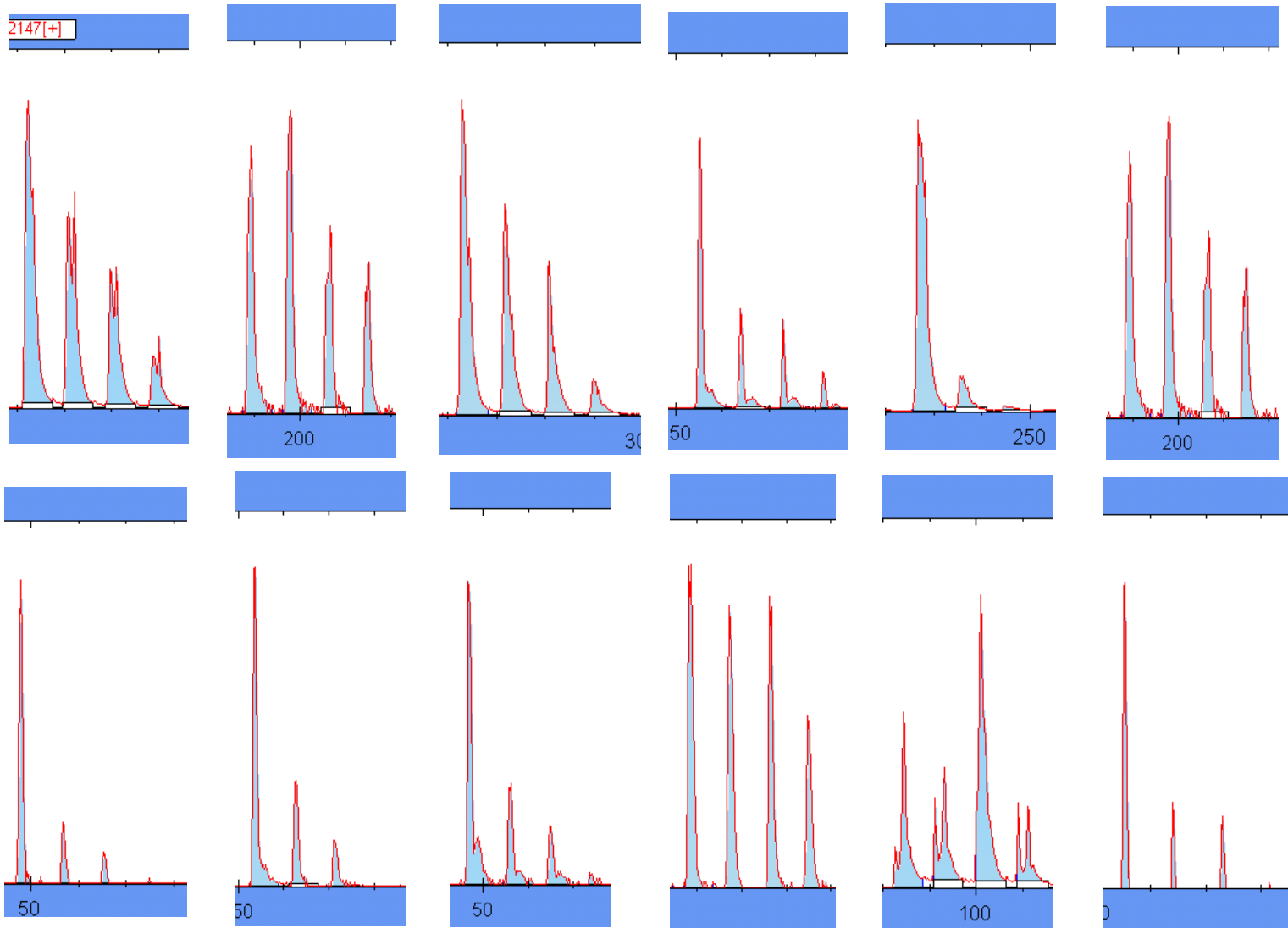
DDI with RapidFire-TSQ Problems and Solutions

Analysis of the OH-metabolite of Substrate

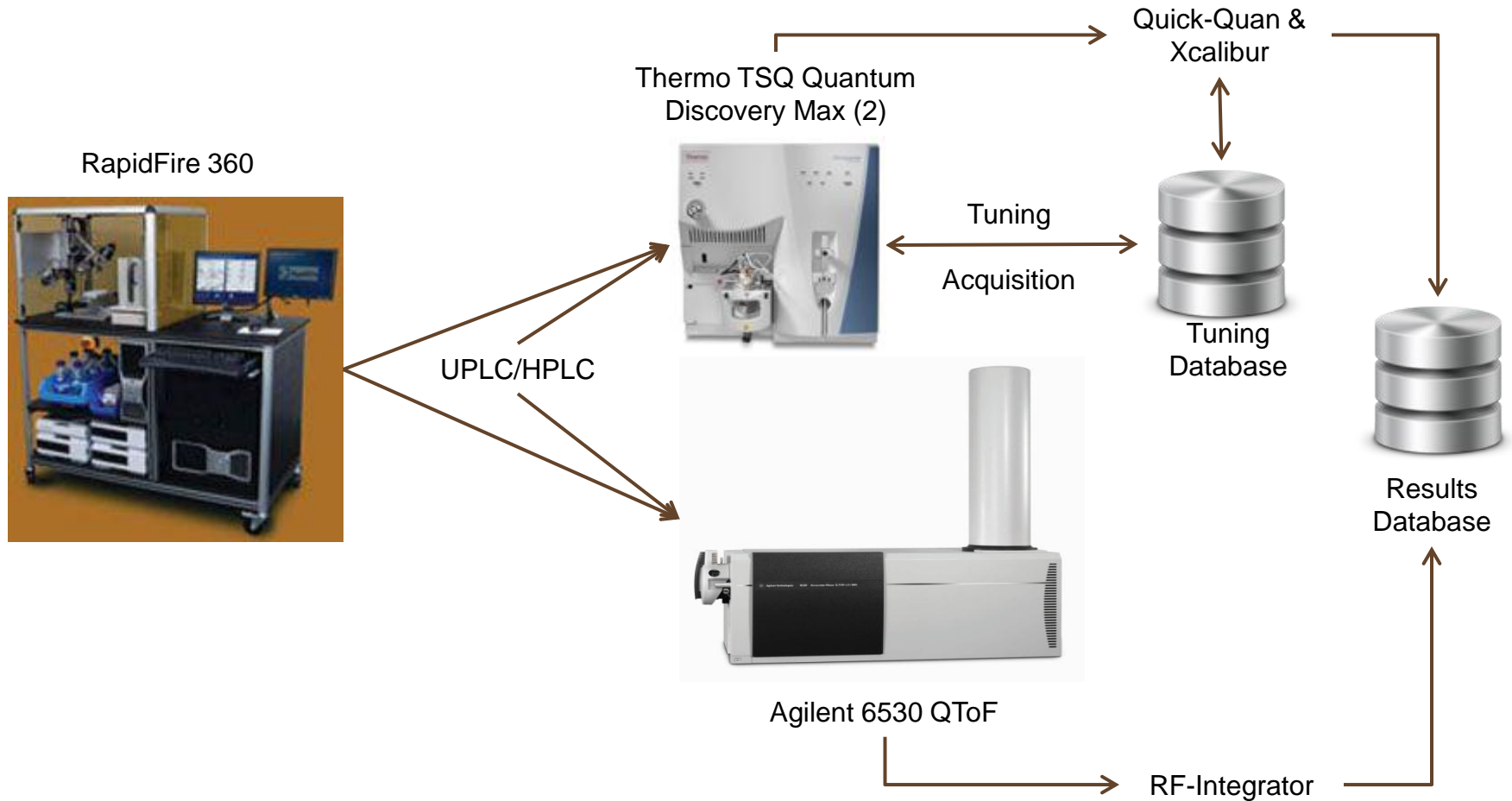
- Low signal of internal standards
 - No pooling of incubations
 - Higher concentration of internal standards
- Integration using the RapidFireIntegrator - skipped
 - Missing sips (not all injections are recorded)
 - “Wrong” Background leads to small peaks integrated as zero (Background is calculated based on the median of the non-selected area)
 - High human impact (export of data from Xcalibur => Excel => txt-file => rawdata folder => import RapidFireIntegrator via USB-stick)
- Integration using Xcalibur
 - Correct alignment by home-made Excel macros
- No communication between RapidFire-TSQ Quantum
 - Synchronizing acquisition times and injection start by contact-closure

The Signals of compounds

Good correlation in spite of very low signals



The setup



Method for TSQ Quantum

General Considerations for the TSQ Quantum

- Run time of 65 min (additional 10 min)
- Reduced scan time of 0.025 s (cycle time of 0.1 s for 4 cmpds)
- Peak width (base) = 3.9 s (FWHM = 1.62 s)
25 data points per peak for the hole peak*
- Higher flow 1 ml/min instead of 300-400 μ l/min requires higher sheet gas flow of 60 arb and capillary 4000 V

* not 39 cause of micro scans