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Experiences with Symbiosis LC-MS/MS

Bioanalysis in the lower pg/mL region in serum and whole blood

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Agenda

- Bioanalysis at Schering AG
- Example 1
- Example 2
- Summary

Bioanalysis at Schering AG

Group within Preclinical development/Pharmacokinetics

- Bioanalysis for preclinical and clinical studies
- Formulation analysis for preclinical studies
- 4 scientists, 14 technicians

Bioanalysis: quantification and metabolite identification

Quantification:

- 2x API365, 2x API3000, 1x API4000, 1x API4000QTrap, 1x ELAN9000

Metabolite ID:

- 1x API3, 1x QTof Ultima Global, 1x LCQ duo, 1x LTQ FT MS

Bioanalysis at Schering AG - quantification

7.75 FTEs dedicated to quantitative bioanalysis

MD/MV per year (per species, matrix)	30 - 50
sample throughput per year	20000

issue: sensitivity versus timelines

LLOQs	< 100 pg/mL
MD/MV per project	3 months

low degree of automatisation, experiences before Symbiosis

- Tecan Genesis for SPE
- Packard Multiprobe for LLE
- Spark Prospect for SPE in metabolism studies
- TFC in metabolism studies

Example 1 - introduction

compound characteristics

- ca. 500 u
- nonpolar (log P_{OW} 4.6)
- insoluble in water at pH 7 and pH 3.7
- pK_a 1.65

development for oral application (dermatology)

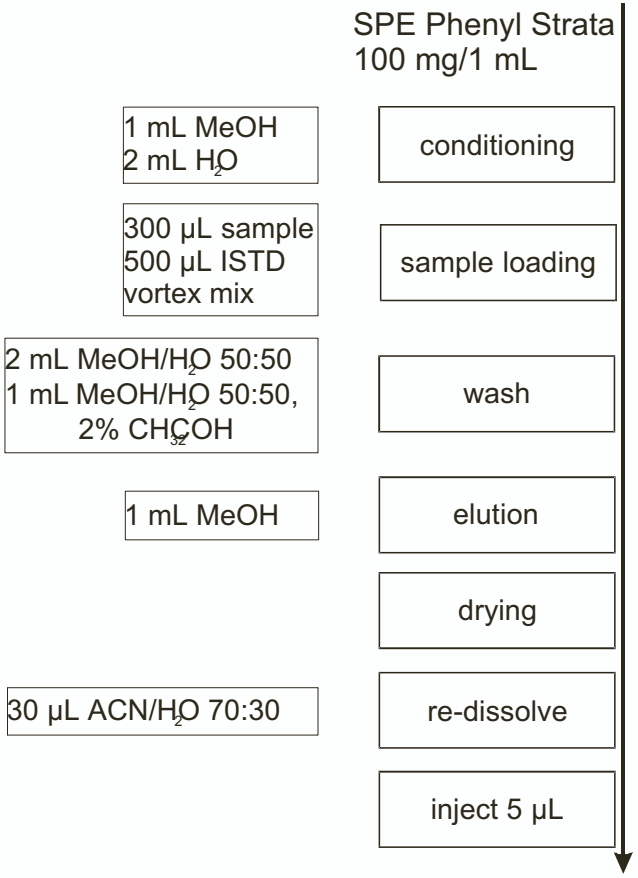
- highly potent - low dosage, low LLOQ (10 pg/mL) in serum
- online design of phase I dose escalation study

experience with Tox species (LLOQ 30 pg/mL)

- problems with chemical interference and matrix effects in serum and plasma
- solved with extensive LC and atmospheric pressure photo-ionisation (APPI) detection
- laborious and time consuming but robust method

Example 1 - manual work-up

SPE



HPLC

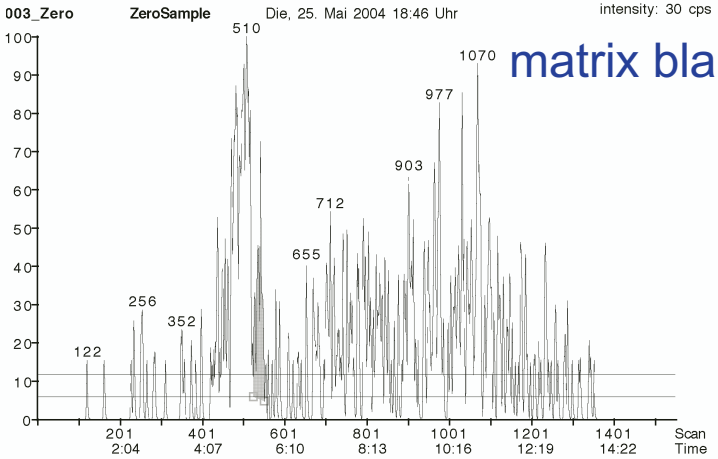
A: water B: ACN

step	time hh:mm:ss	A [%]	B [%]	Flow [mL/min]
1	00:00:00	70	30	0.2
2	00:08:00	30	70	0.2
3	00:08:10	0	100	0.2
4	00:12:00	0	100	0.2
5	00:12:10	70	30	0.2
6	00:17:00	70	30	0.2

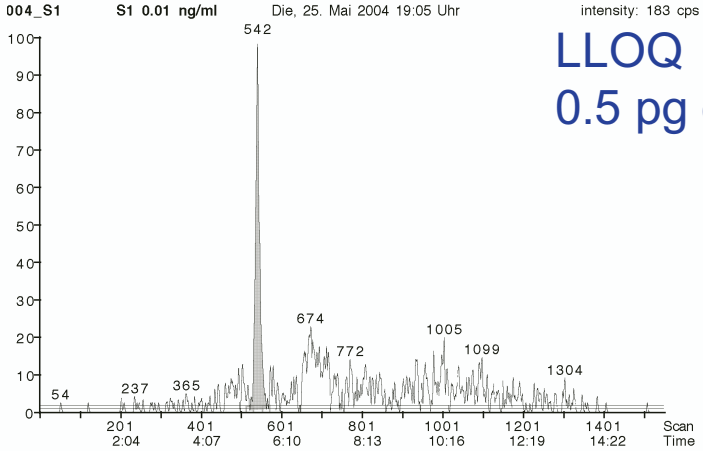
Detection

+ve APPI API3000

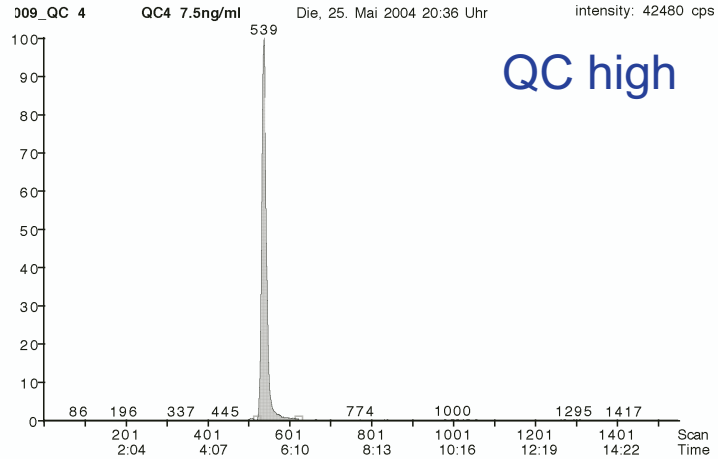
Example 1 - manual work-up, chromatograms



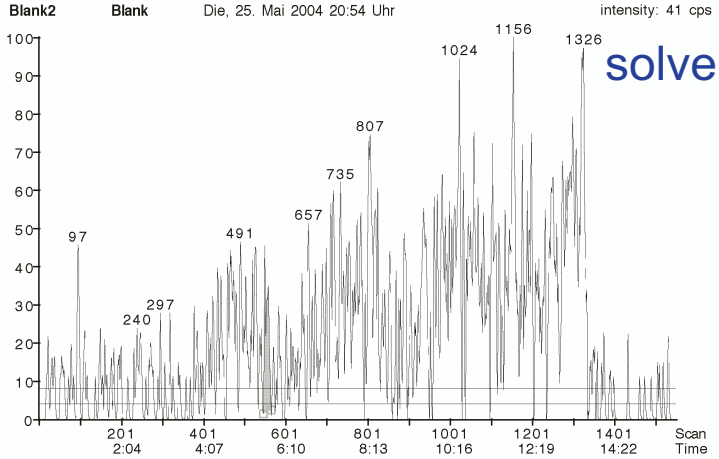
matrix blank



LLOQ
0.5 pg on column



QC high



solvent blank

sufficient sensitivity, no carry-over

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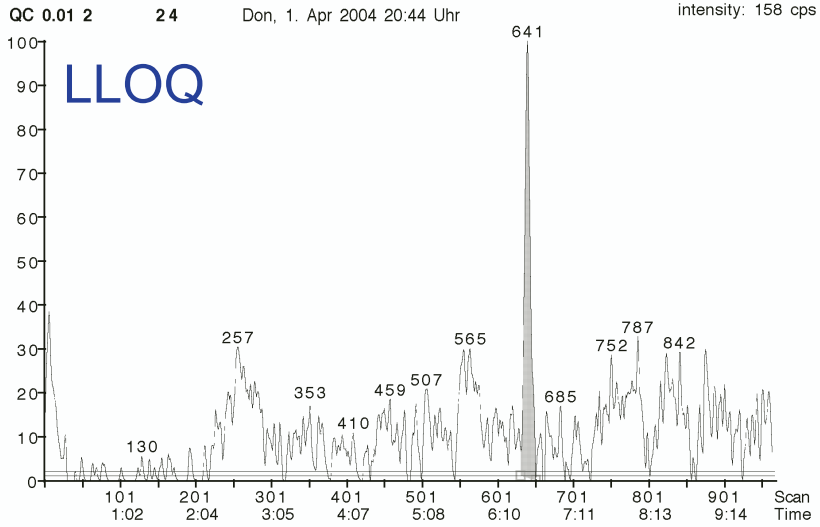
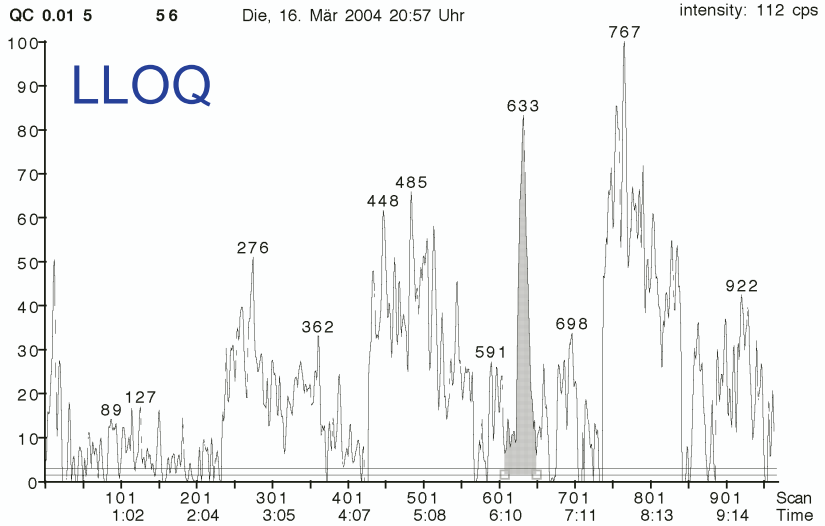
Example 1 - manual work-up vs. Symbiosis

		SPE Phenyl Strata 100 mg/1 mL	HySphere Resin GP 10-12µm		
	conditioning	1 mL MeOH 2 mL H_2O	solvation equilibration	1 mL MeOH 1 mL H_2O	
300 µL sample 500 µL ISTD vortex mix	sample loading		sample loading	250µL partial loopfill	350 µL sample 30 µL ISTD vortex mix
			sample extraction	1 mL H_2O	
2 mL MeOH/H_2O 50:50 1 mL MeOH/H_2O 50:50, 2% CH_3COOH	wash		wash	2 mL H_2O	
	elution	1 mL MeOH	elution	0.2 mL ACN	
	drying				
	re-dissolve	30 µL ACN/H_2O 70:30			
	inject 5 µL		injection		

step	time hh:mm:ss	A [%]	B [%]	Flow [mL/min]	
1	00:00:01	100	0	0.2	focussing
2	00:02:00	100	0	0.2	focussing
3	00:02:01	70	30	0.2	
4	00:05:00	30	70	0.2	
5	00:06:00	0	100	0.2	
6	00:09:00	0	100	0.2	
7	00:09:01	70	30	0.2	
8	00:12:01	100	0	0.2	

Example 1 - Symbiosis work-up, overall sensitivity

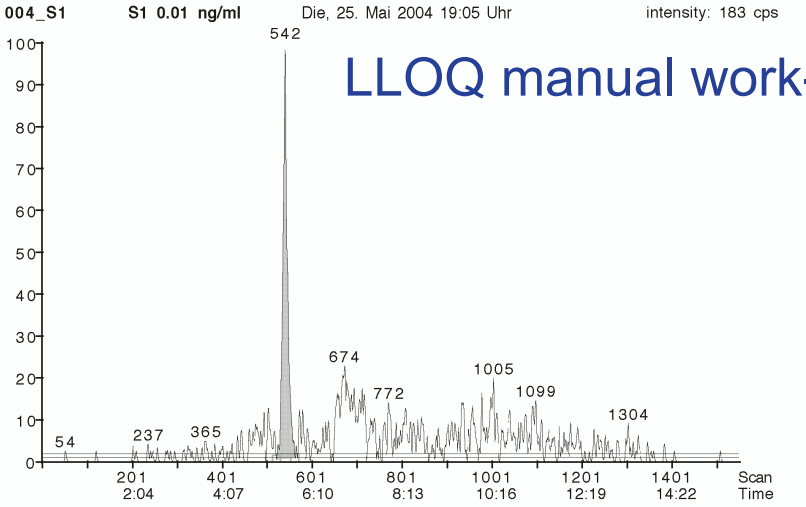




2.3 pg on column

Comparison with manual work-up:
0.5 pg on column
ca. 5 times more sensitive

Note: modified LC method had no influence



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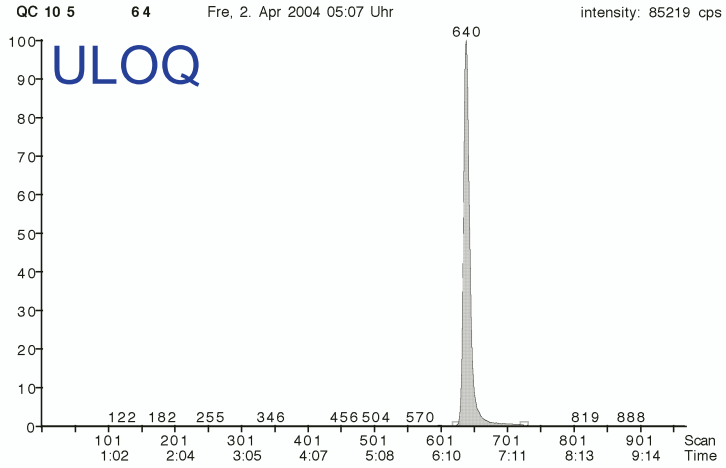
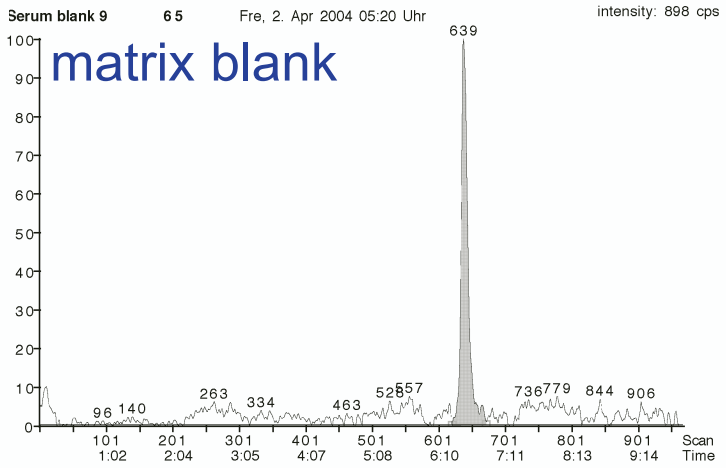
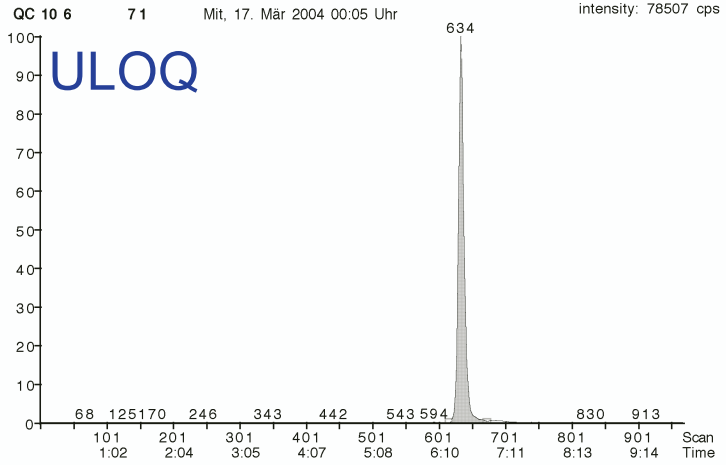
Example 1 - Symbiosis work-up, carry-over

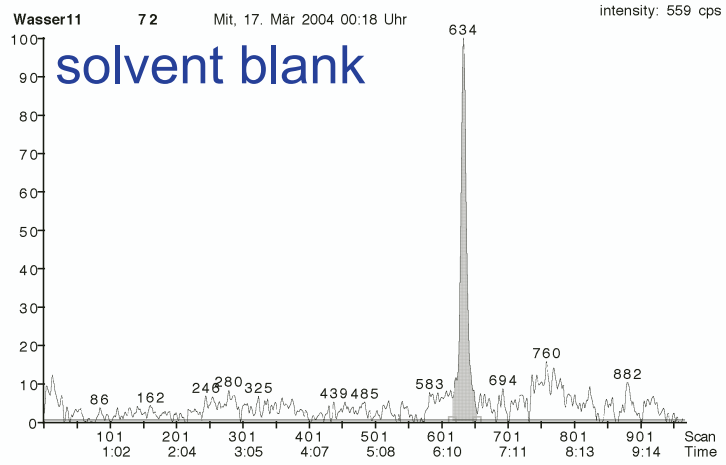




Carry over [%]

1.1





0.7

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Example 1 - intermediate results

Method does not reach desired sensitivity

- method optimisation with regard to
  - extraction efficiency
  - matrix interference

Method has unacceptable carry-over

- carry-over is linear throughout concentration range:

STD conc [ng/mL]	area	solvent blank area	carry-over [%]
1	66538	564	0.8
2.5	146314	1355	0.9
7.5	418459	4239	1.0
10	474286	4176	0.9

- reduction of calibrated range to e.g. 0.01 - 1 ng/mL not possible

further method optimisation and wash programs...

Example 1 - method optimisation

HySphere C18 HD 7µm

1 mL MeOH 1 mL H_2O	solvation equilibration
350 µL sample 30 µL ISTD vortex mix	250µL partial loopfill → sample loading
0.5 mL H_2O	sample extraction
2 mL H_2O	wash
0.2 mL ACN/H_2O 90:10	elution
	injection

Autosampler program
SparkLink 3.9

Line	Wash Vol	SSV port
1	700 µL	2
2	700 µL	3
3	700 µL	2

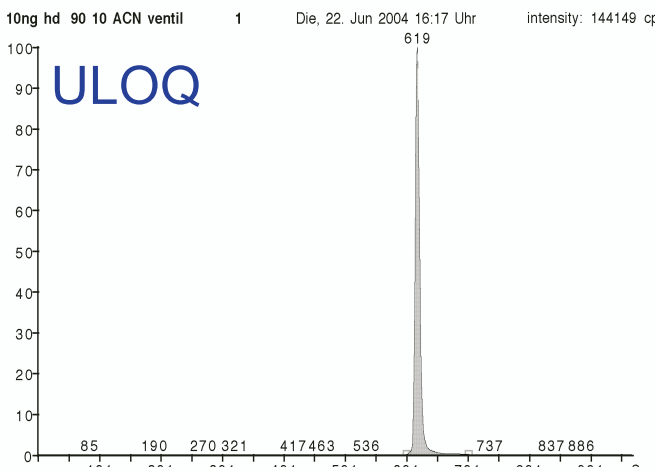
2 MeOH/H2O 10:90
3 MeOH/0.1% HCO2H 50:50

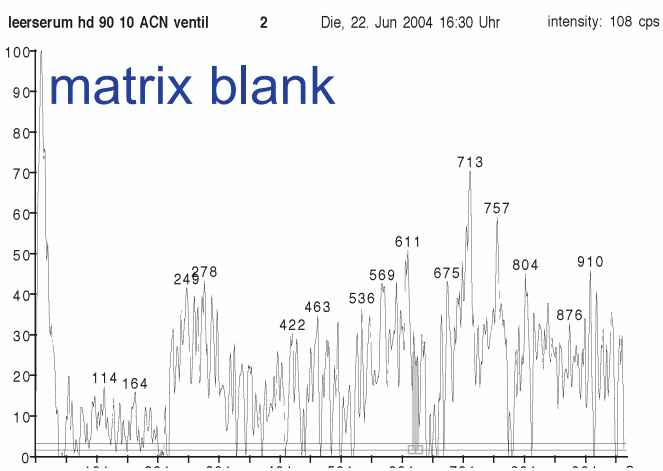
clamp flush 0.5 mL H_2O	wash cartridge 3 mL ACN/H_2O 90:10
	clamp flush 0.5 mL ACN/H_2O 90:10 0.5 mL H_2O

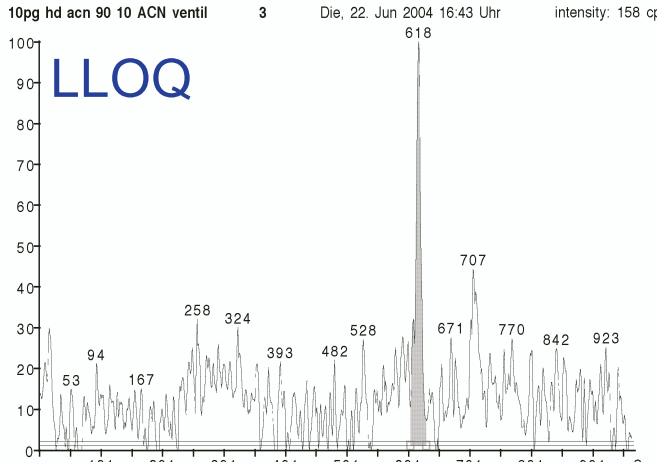
step	time hh:mm:ss	A [%]	B [%]	Flow [mL/min]	
1	00:00:01	90	10	0.2	focussing
2	00:02:00	90	10	0.2	focussing

Example 1 - successful elimination of carry-over

1	Wait for Input	2-LOW			
2	Valve	ISS-A 1-2			
3	Wait for Input	3-LOW			
4	Syringe Valve	SOLVENT 3			
5	Wait	00:00:06			
6	Valve	INJ Load			
7	Wash	500 µl			
8	Valve	INJ Inject			
9	Wash	500 µl			
10	Valve	INJ Load			
11	Wash	500 µl			
12	Valve	INJ Inject			
13	Wash	500 µl			
14	Syringe Valve	SOLVENT 2			
15	Valve	INJ Load			
16	Wash	500 µl			
17	Valve	INJ Inject			
18	Wash	500 µl			
19	Valve	INJ Load			
20	Wash	500 µl			
21	Valve	INJ Inject			
22	Wash	500 µl			
23	Syringe Valve	SOLVENT 3			
24	Valve	INJ Load			
25	Wash	2500 µl			
26	Valve	INJ Inject			
27	Aspirate	90 µl	From: Sample	Speed: 3	Height: 5
28	Valve	INJ Load Sync			
29	Aspirate	250 µl	From: Sample	Speed: 3	Height: 5
30	Valve	INJ Inject Sync			
31	Marker	INJECT			
32	Dispense	340 µL	To: Waste	Speed: 6	Height: 0
33	Syringe	HOME			
34	Syringe Valve	SOLVENT 3			
35	Syringe	LOAD	250 µl	Speed: 6	
36	Syringe Valve	NEEDLE			
37	Dispense	250 µl	To: Waste	Speed: 6	Height: 0
38	Syringe Valve	SOLVENT 3			
39	Wash	1000 µl			
40	End				







But no improvement of sensitivity

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Example 2 - introduction

Compound characteristics

- ca. 450 u
- polar (log P_{OW} 1.29 at pH 7, determined by HPLC)
- insoluble in water at pH 7, slightly soluble at pH 1.1 and in organic solvents
- pK_a 4.0, 10.3

development for oral application (oncology)

- whole blood analysis (+ plasma and urine)
- Phase I concentration range approx. 100 pg/mL - 1 µg/mL

experience with Tox species (LLOQ 5 ng/mL)

- problems with sticky behaviour and sample matrix
- solved with extensive sample preparation and +ve ESI detection
- laborious and time consuming but robust method

Example 2 - development of two methods

Split of concentration range:

5.0 ng/mL - 1.0 µg/mL

0.1 ng/mL - 10 ng/mL

development of method for the high concentration range in analogy to manual work-up method

Example 2 - sample preparations, LLOQ 5 ng/mL

manual

SPE Oasis HLB
1cc (30mg)

2 mL MeOH 2 mL H_2O	conditioning
50 µL sample 100 µL ISTD 1200 µL buffer vortex mix centrifuge	sample loading
2 mL H_2O 1 mL 5% MeOH 1 mL MeOH/2% NH_4OH 40:60 1 mL H_2O 1 mL MeOH/2% CH_3COH 40:60	wash
1.4 mL MeOH/H_2O 70:30	elution
	drying
25 µL MeOH/H_2O 50:50	re-dissolvation
	injection 2 µL

Symbiosis

HySphere Resin GP
10-12µm

solvation equilibration	1 mL MeOH 1 mL 5% MeOH
sample loading	50µL partial loopfill vortex mix centrifuge 50 µL sample 50 µL ISTD 1900 µL buffer
sample extraction	0.5 mL 5% MeOH
wash	1 mL 5% MeOH 1 mL MeOH/2% NH_4OH 40:60 1 mL 5% MeOH 1 mL MeOH/2% CH_3COH 40:60 1 mL 5% MeOH
elution	0.2 mL MeOH/H_2O 70:30
injection	

API365

time hh:mm:ss	A [%]	B [%]	Flow [mL/min]
00:00:01	75	25	0.3
00:01:00	75	25	0.3
00:05:00	25	75	0.3
00:05:20	0	100	0.5
00:06:00	0	100	0.5
00:06:20	75	25	0.5
00:09:00	75	25	0.3

API4000

time hh:mm:ss	A [%]	B [%]	Flow [mL/min]
00:00:01	90	10	0.35
00:02:00	90	10	0.35
00:02:05	75	25	0.30
00:03:00	75	25	0.30
00:07:00	25	75	0.30
00:07:12	0	100	0.50
00:09:00	0	100	0.50
00:11:00	90	10	0.35

Example 2 - Symbiosis wash programs

HySphere Resin GP
10-12µm

solvation equilibration	1 mL MeOH 1 mL 5% MeOH		
sample loading	50µL partial loopfill	vortex mix centrifuge	50 µL sample 50 µL ISTD 1900 µL buffer
sample extraction	0.5 mL 5% MeOH		
wash	1 mL 5% MeOH 1 mL MeOH/2% NH_4OH 40:60 1 mL 5% MeOH 1 mL MeOH/2% CH_3CO_2H 40:60 1 mL 5% MeOH		
elution	0.2 mL MeOH/H_2O 70:30		
injection			

Autosampler program
SparkLink 3.10

valve wash
2 mL MeOH

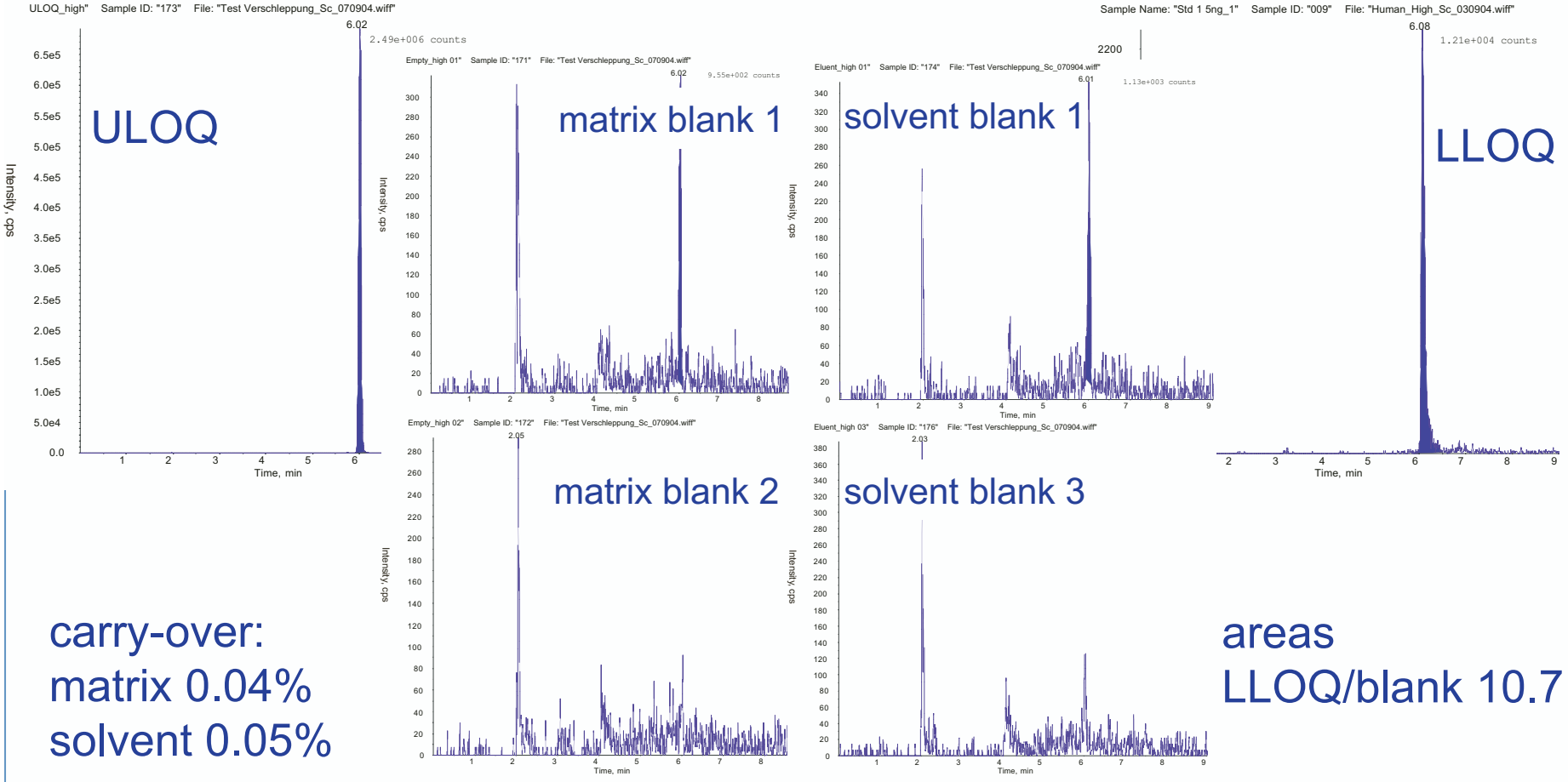
clamp flush
0.5 mL MeOH/H_2O 70:30
0.5 mL H_2O
0.5 mL MeOH/H_2O 70:30

Line	Wash Vol	SSV port	Valve wash
1	1000 µL	1	No
2	1000 µL	2	No
3	1000 µL	1	No
4	1000 µL	1	Yes
5	1000 µL	2	Yes
6	1000 µL	3	Yes
7	1000 µL	1	Yes
8	1000 µL	1	Yes
9	1000 µL	1	Yes

1 5% MeOH
2 MeOH
3 MeOH/2% CH3CO2H 40:60

Example 2 - comparison of LLOQ 5 ng/mL methods

Manual work-up: carry-over 0.09%; area LLOQ/ area blank 11.2



carry-over:
matrix 0.04%
solvent 0.05%

areas
LLOQ/blank 10.7

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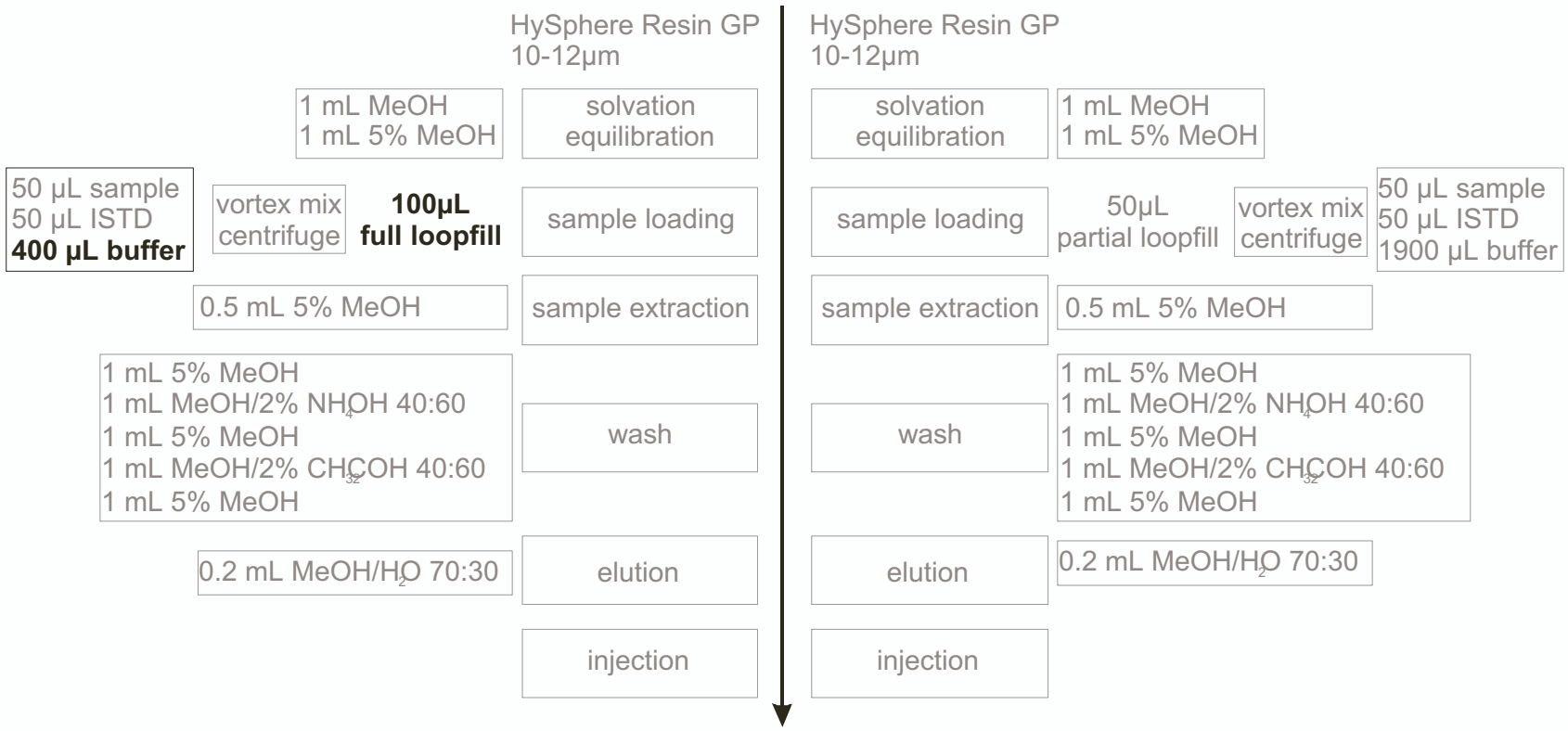
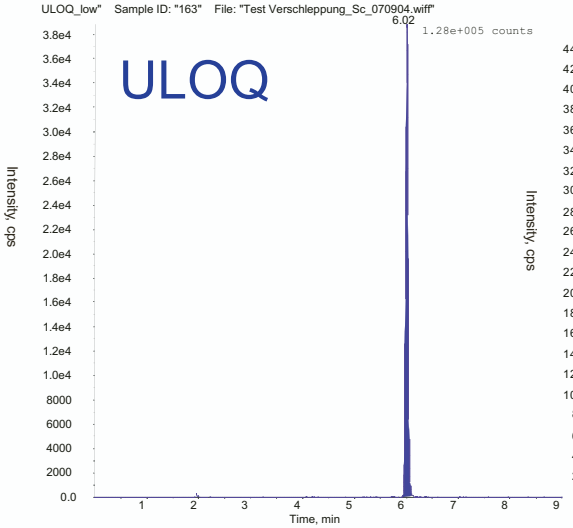
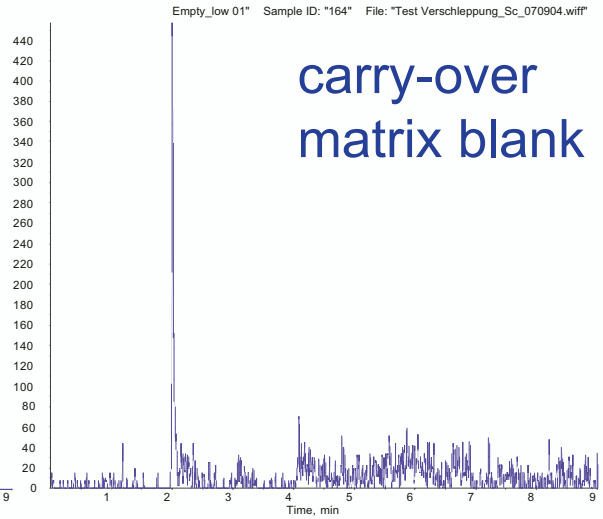
Example 2 - method adjustment for LLOQ 100 pg/mL



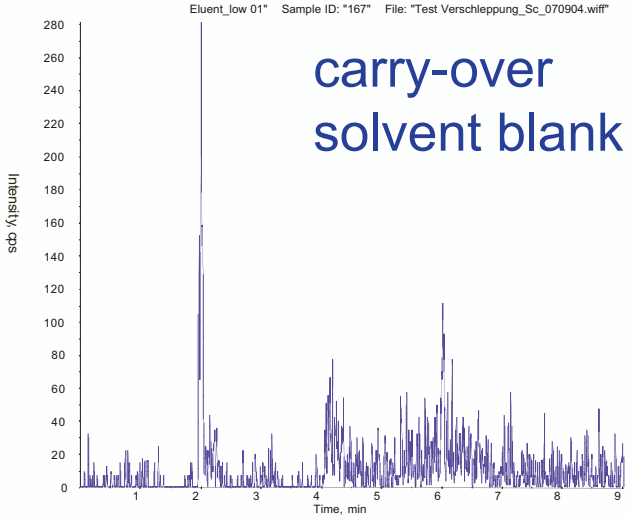
Example 2 - results for LLOQ 100 pg/mL method



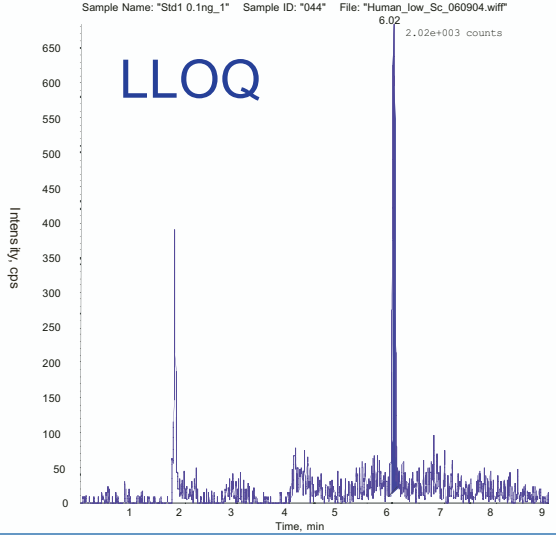
ULOQ



carry-over matrix blank



carry-over solvent blank



LLOQ

Summary

- easy method adjustment for lower LLOQ
- carry-over concentration dependent?

Further considerations and summary

Method transfer time:

- from March to September 2004 (2 people, not full-time)
- In the beginning we needed support from Spark (for the autosampler program) - this was granted generously.
- With the first Symbiosis Pharma system we initially had some problems with the system performance. However, after an upgrade program from Spark these performance problems were solved.

Apart from that:

- The system is suited for our purpose (low LLOQs, low carry-over), but low LLOQs need more than average method development capacities
- SparkLink 3.10 helped a lot in reducing carry-over, remaining substance may derive from the LC connection (T-piece for focussing mode)

Thanks

Spark Holland: everybody who helped, especially Martin Sibum

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The group

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Petra Pietzuch