



Cell-Disintegrated Blood (CDB): A unique substitute for Dried Blood Spots (DBS)

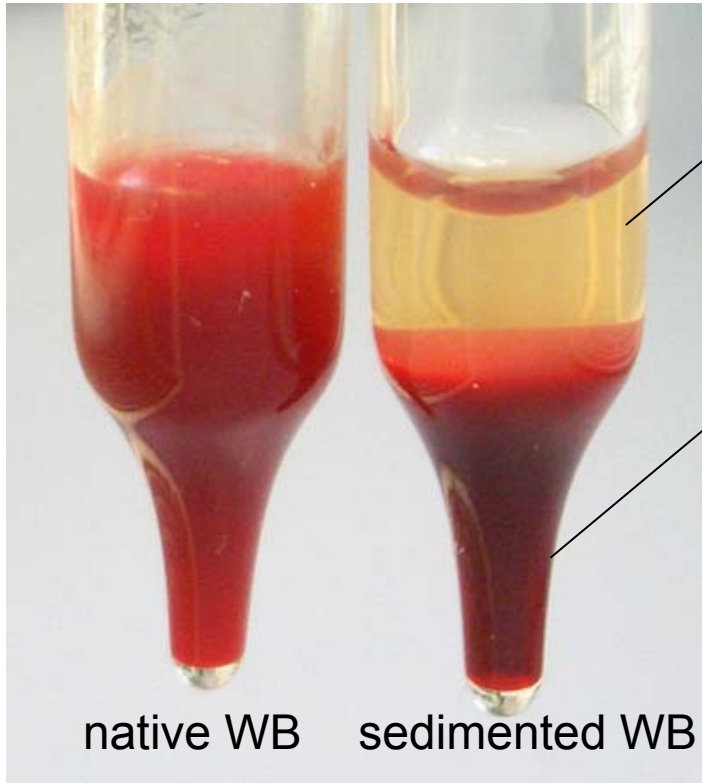
Karl-Siegfried Boos

Laboratory of BioSeparation
Institute of Clinical Chemistry
Medical Center of the University of Munich
Munich, Germany

8. LC/MS-Diskussionstreffen
April 20, 2010, Wuppertal, Germany



Components of Whole Blood

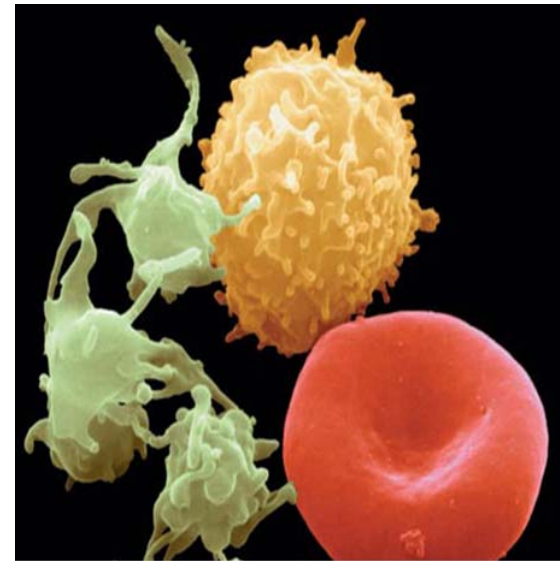


Plasma

Blood cells

native WB

sedimented WB



Leucocyte

8-20 μm

7.1×10^3 per μL

Erythrocyte

7.5 μm

5×10^6 per μL

Thrombocytes

2-3 μm

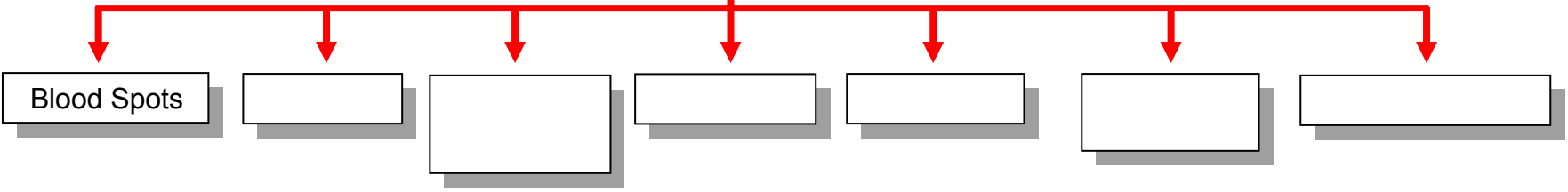
2.5×10^5 per μL

© Max-Planck-Gesellschaft

Hematocrit (Hct) : volume fraction of erythrocytes

Processing of **Whole Blood**

Manual (off-line) / Robotic (at-line)



LC-MS/MS

Dried Blood Spots (DBS)

Sample Capillary blood

Sampling site

- A) Heel
- B) Fingerprick

Sampling device Filter card

Applications

- Newborn Screening
- Toxicological Studies (Phase I)
- Therapeutic Drug Monitoring (e.g. antiretrovirals, antimalarials, immunosuppressants)

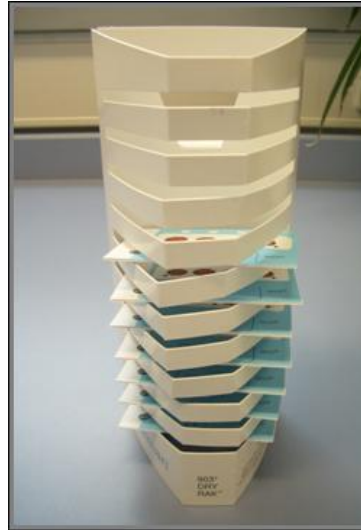


Dried Blood Spots : Analysis of Immunosuppressants

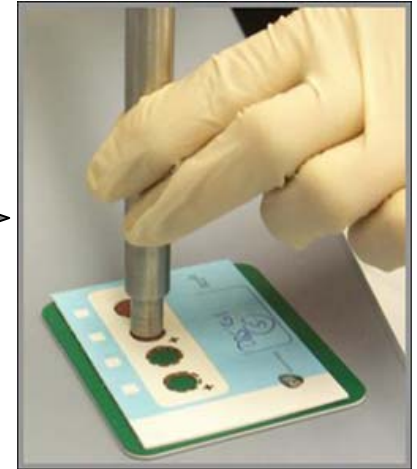
Hoogtanders K. et al. *J Pharm Biomed Anal* 44 (2007) 658-664



Pipette 30 μL of whole blood and deposit onto filter card



Dry at RT (> 3 hrs)



Punch disc ($r = 3.75 \text{ mm}$)



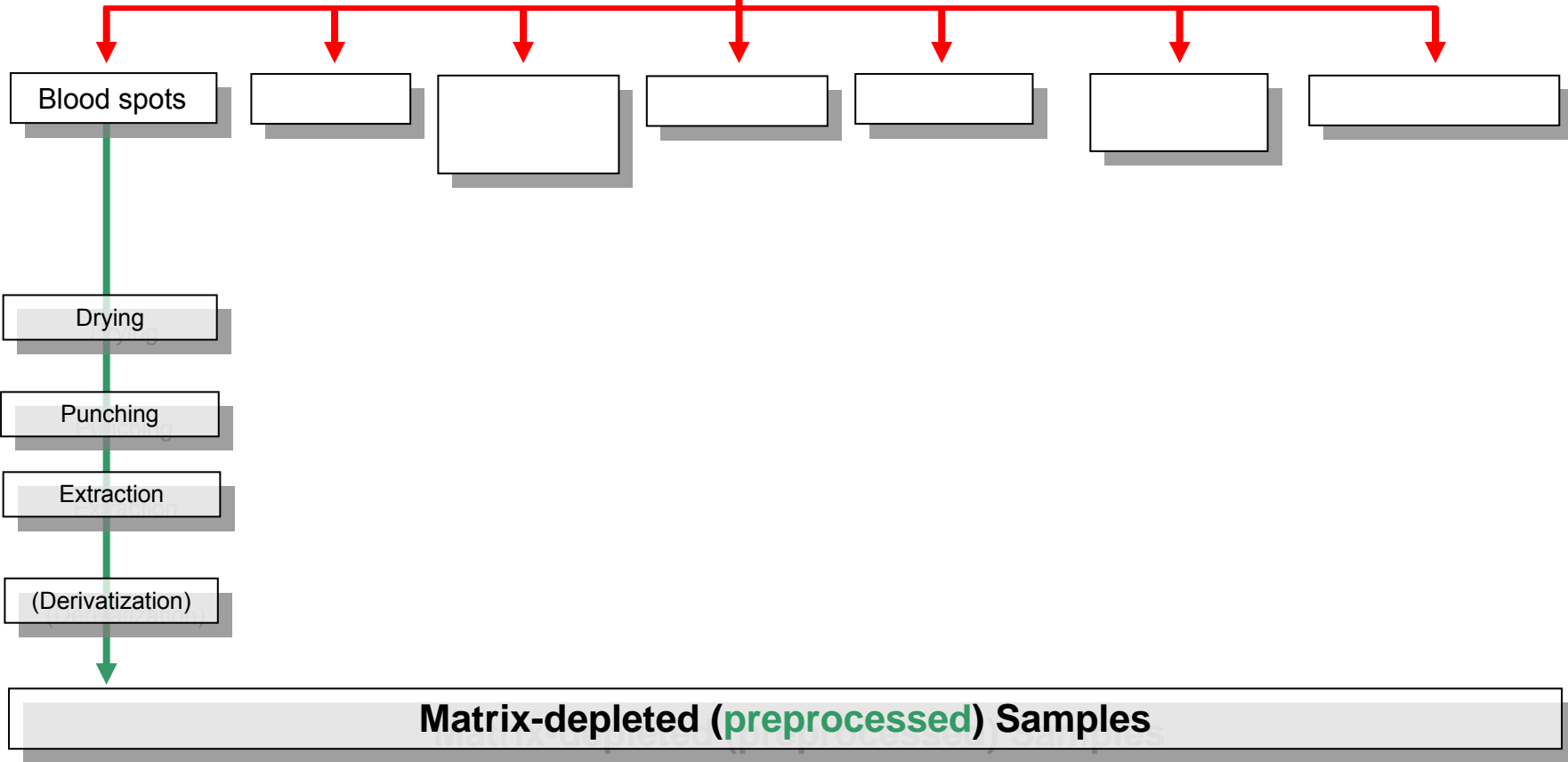
Extract disc with 250 μL organic solvent containing internal standard (horizontal shaking for 60 min)

Pipette aliquot of extract into vial

Inject 25 μL onto LC-MS/MS

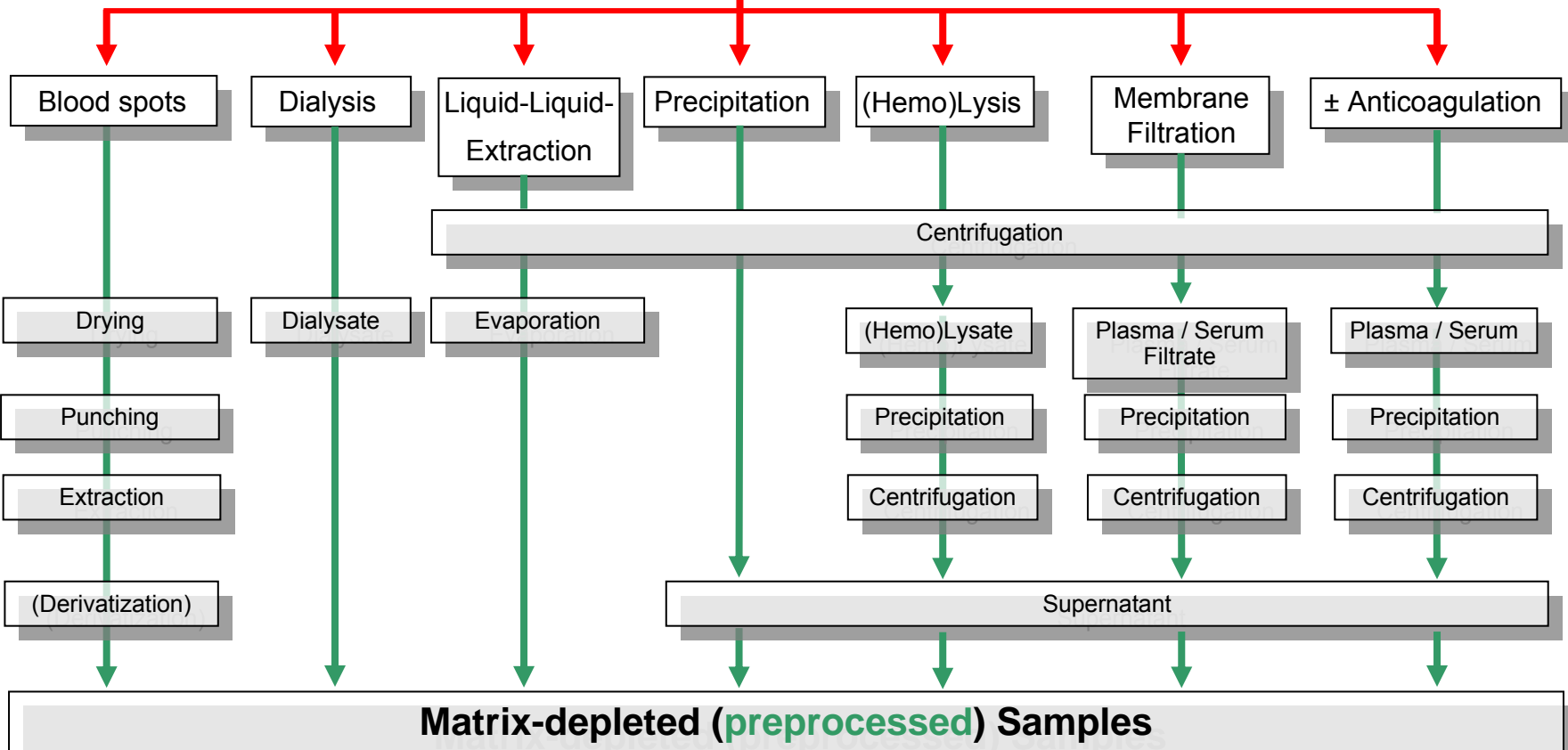
Processing of Whole Blood

Manual (off-line) / Robotic (at-line)



Processing of **Whole Blood**

Manual (off-line) / Robotic (at-line)

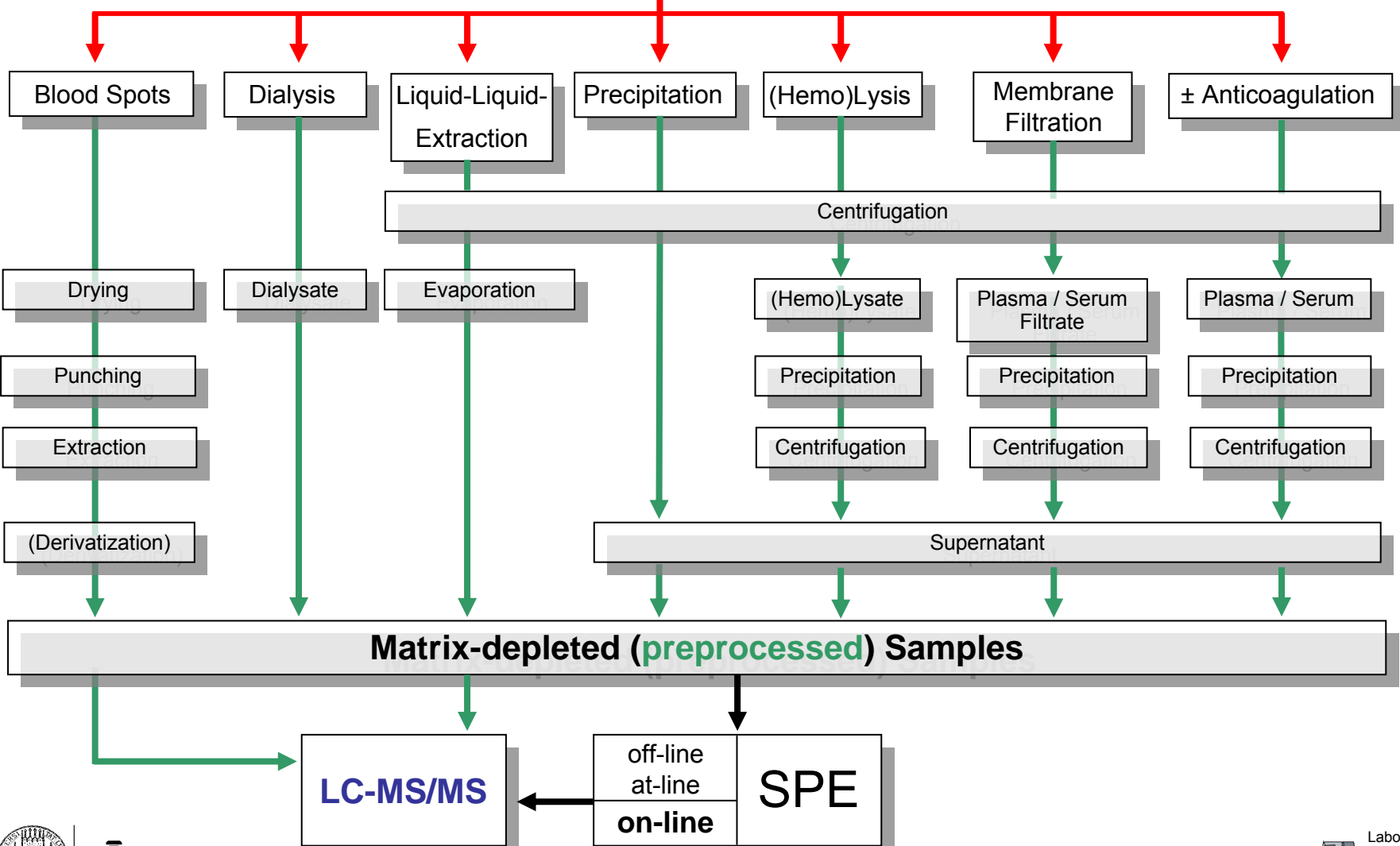


Solid Phase Extraction (SPE)



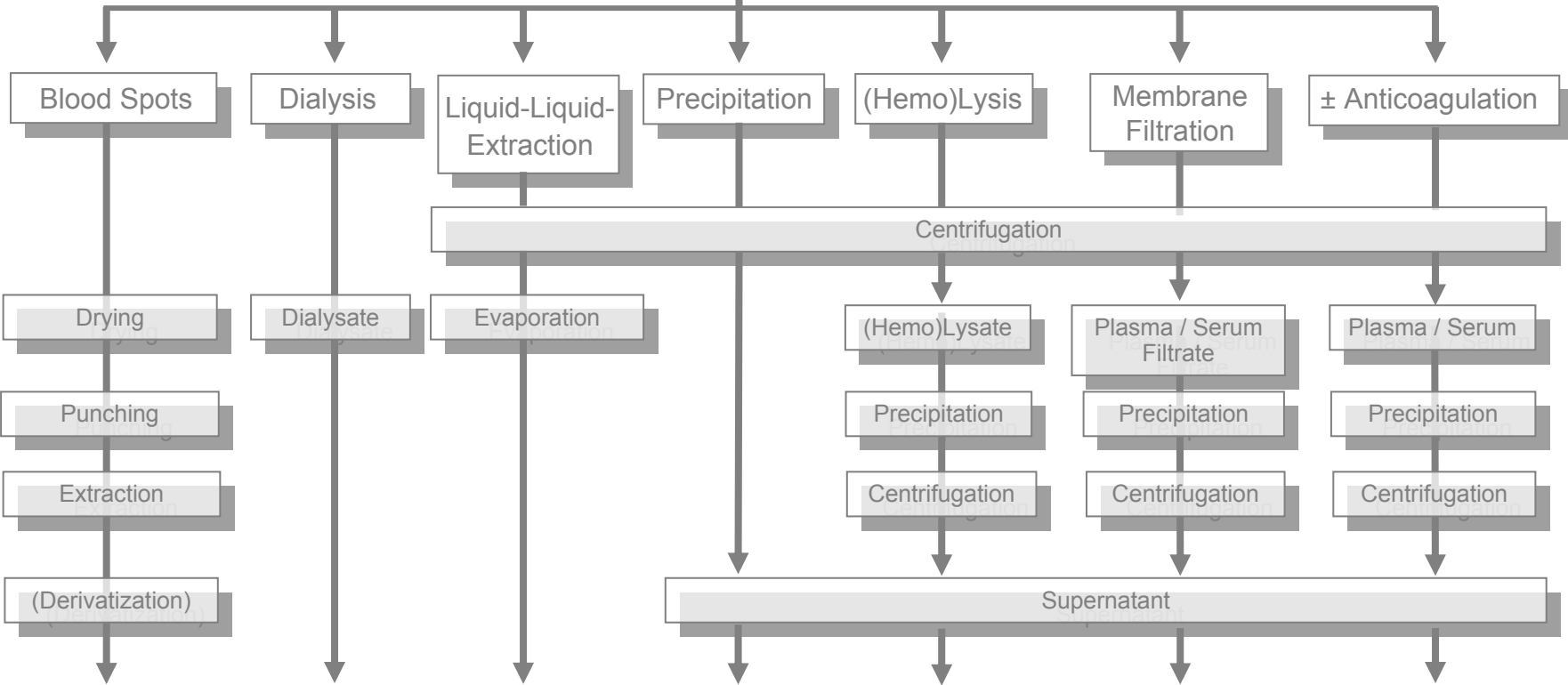
Processing of Whole Blood

Manual (off-line) / Robotic (at-line)



Processing of **Whole Blood**

Manual (off-line) / Robotic (at-line)



Matrix-depleted (preprocessed) Samples

LC-MS/MS

off-line
at-line
on-line **SPE**

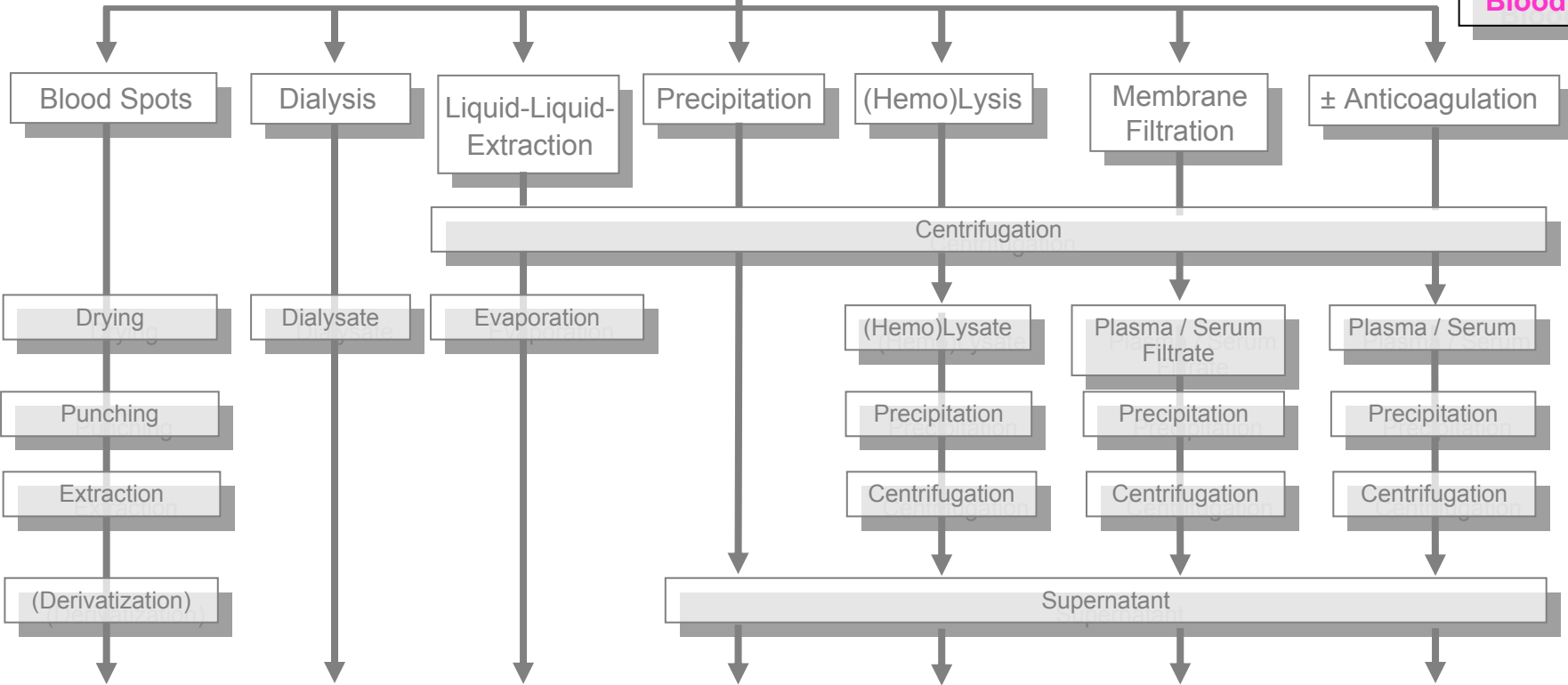
Matrix-containing (native) Samples

Processing of Whole Blood

in-line

Manual (off-line) / Robotic (at-line)

Cell-disintegrated Blood (CDB)



Matrix-depleted (preprocessed) Samples

LC-MS/MS

off-line at-line on-line SPE

Matrix-containing (native) Samples

Conversion of whole blood into CDB

A) BloodLyser[®]

- A sedimented sample of anticoagulated **blood** is **remixed** before in-line treatment.
- To an aliquot of the homogenized blood sample an **internal standard** (IS), dissolved in DMSO, is added and the sample is vortexed again.
- An aliquot of the blood sample then is processed by pumping it through a **heated stainless steel capillary** (BloodLyser[®]).

The processing conditions are chosen in such a way that the resulting **heat-shock** causes **no precipitation** of blood proteins.

The blood cells become **disintegrated** by this treatment and the resulting membrane fragments finally form subcellular particles.

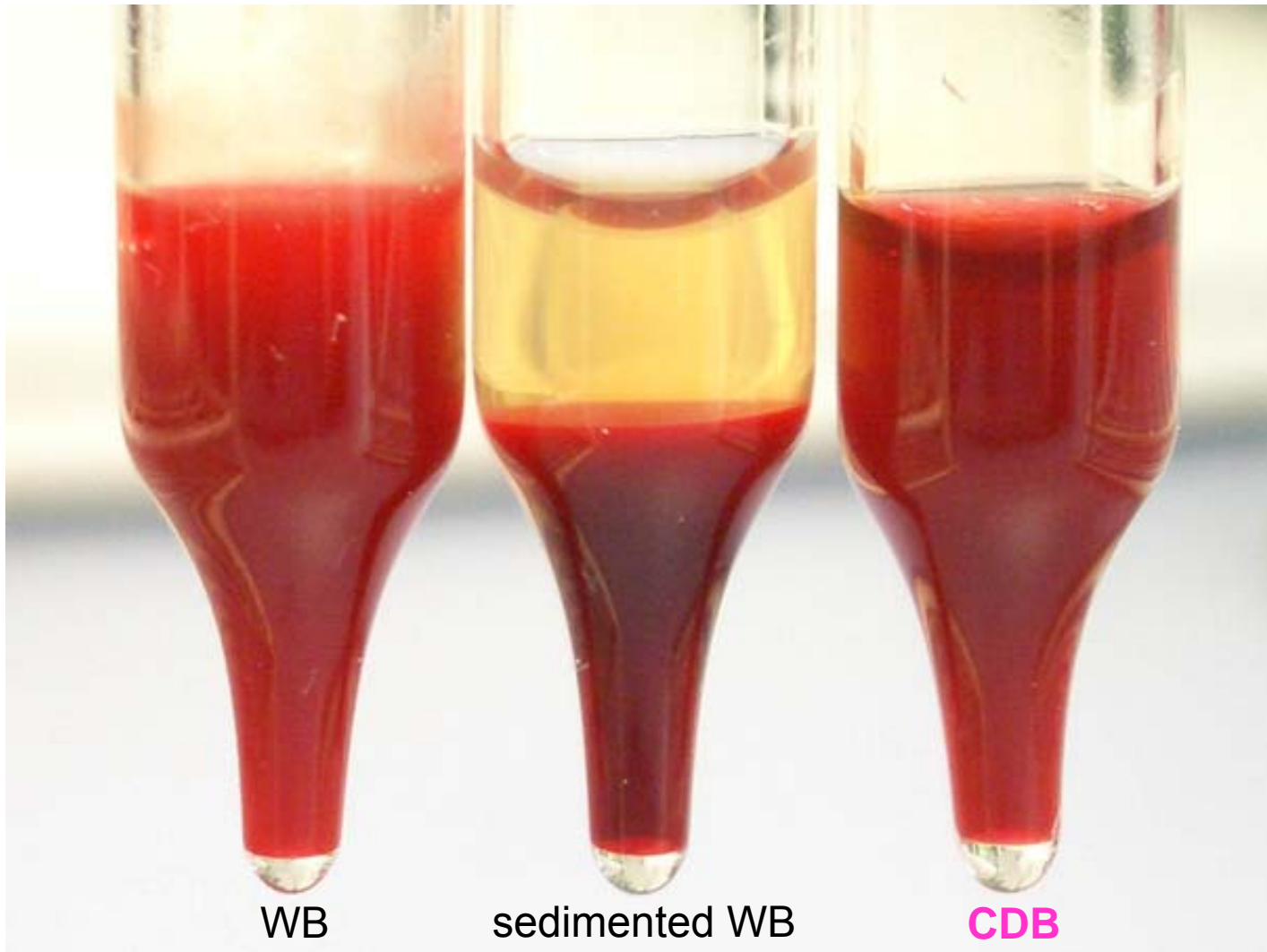


Generation of a new matrix named
Cell-Disintegrated Blood (CDB)

Morello R, Milojkovic J, Boos K-S, Ther Drug Monit 29 (2007) 505



Heat-shock induced conversion of whole blood (WB) into cell-disintegrated blood (CDB)



WB

sedimented WB

CDB

Blood Cell Count :

Before and after heat-shock treatment

Blood Cells	no treatment		heat-shock treatment	
	Number [μL^{-1}]	Percentage [%]	Number [μL^{-1}]	Percentage [%]
Erythrocytes	5×10^6	95.12	0	0
Leucocytes	7.1×10^3	0.13	0	0
Thrombocytes	2.5×10^5	4.75	1.25×10^5	2.38
Total	5.26×10^6	100	1.25×10^5	2.38

Processing temperature : 75°C

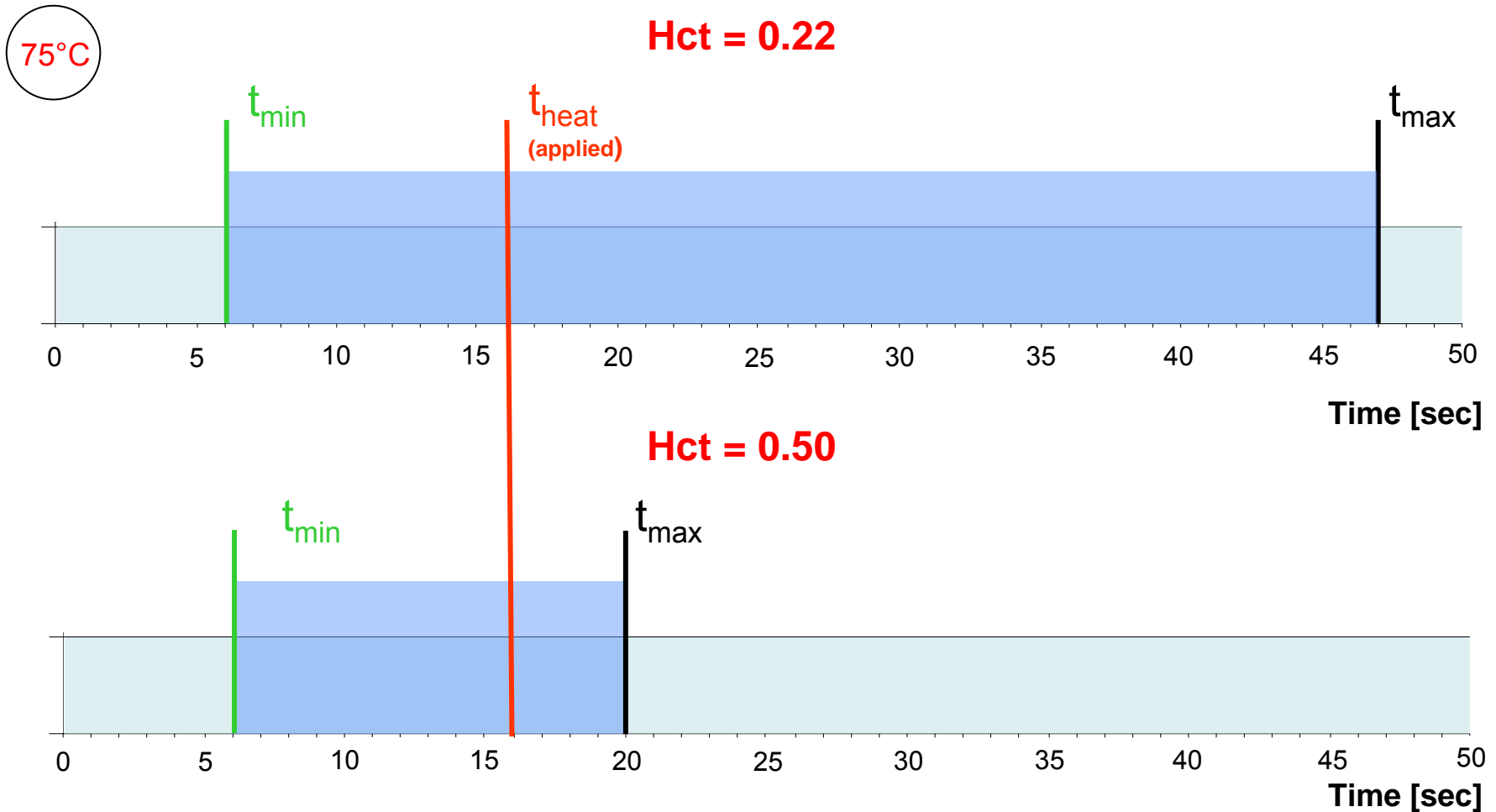
$t_{\text{heat(applied)}}$: 16 sec

Sample : 10 μL whole blood (Hct : 0.40)



Preparation of CDB

Influence of hematocrit (Hct) on process parameters



Samples : anticoagulated whole blood

Preparation of CDB

- Do the process parameters (t_G , t_{max} , t_{min} , t_{heat}) depend on the species the blood sample is taken from ?

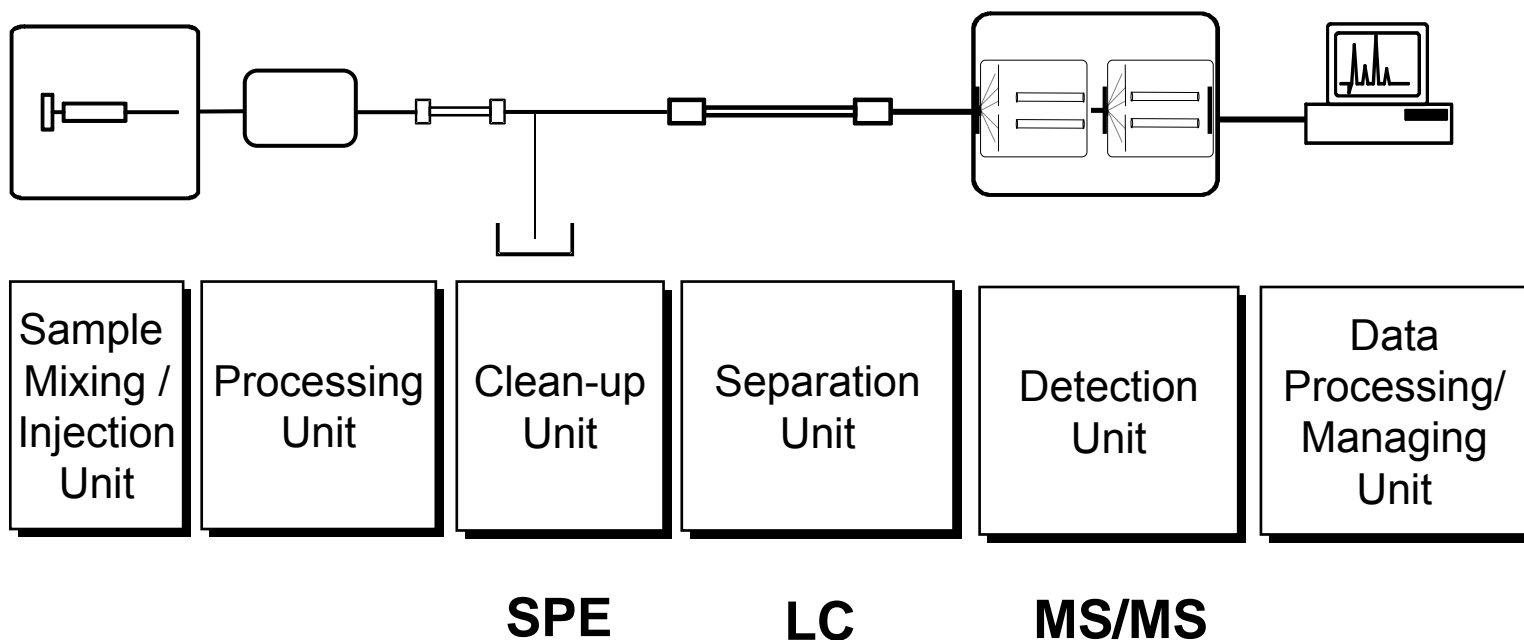
species	n	Hct	t_{max} [sec] at 75°C	t_{min} [sec] at 75°C
human	1	0.40	26	6
rat (Wistar)	1 -2	0.36	23	7
rat (Dark Agouti)	1	0.35	30	7
rat (Piebald-Virol-Glaxo)	2	0.33	41	6
rat (Sprague Dawly)	5	0.39 - 0.45	36 - 42	2 - 3
mice	2	0.34	18	7
guinea pig	1	0.35	17	7
cord blood	2	0.46 – 0.55	29 – 37	5 - 14
dog (Beagle)	1	0.58	31	7

Hct = hematocrit

$t_{heat\ applied} = 16\ sec$

Set-up of a Total Analysis System (TAS) :

In-line processing of whole blood
coupled to on-line SPE-LC-MS/MS analysis



Instrumentation for in-line processing of whole blood

A) BloodLyser[®]

- Bar code reader
- Over-head shaker
- Heated capillary (HotCap[™])



Autosampler

on-line SPE



Integrity[™]

ACE

In cooperation with Spark Holland B.V. and EUREKA (Project E!4112)

Conversion of whole blood into CDB

B) BloodIcer[®]

- A sedimented sample of anticoagulated **blood** is **remixed** before in-line treatment.
- To an aliquot of the homogenized blood sample an **internal standard** (IS), dissolved in DMSO, is added and the sample is vortexed again.
- An aliquot of the spiked sample is aspirated into a hollow stainless-steel needle. Then, the needle is immersed into **liquid nitrogen** whereby the blood sample is **snap-frozen**.
- After the **cryogenic treatment** (BloodIcer[®]) the sample is **thawed** and yields **Cell-Disintegrated Blood (CDB)**.



Instrumentation for in-line processing of whole blood

B) Bloodlcer®

- Bar code reader
- Vortexer / Heater
- Container for liquid nitrogen



In cooperation with LEAP Technologies, USA

Application of CDB : Therapeutic Drug Monitoring (TDM)

- Drugs predominantly bound to blood cells
e.g. immunosuppressants bound to erythrocytes
- Drugs predominantly bound to proteins present in blood plasma
e.g. antimycotics



Analysis of drugs in CDB :

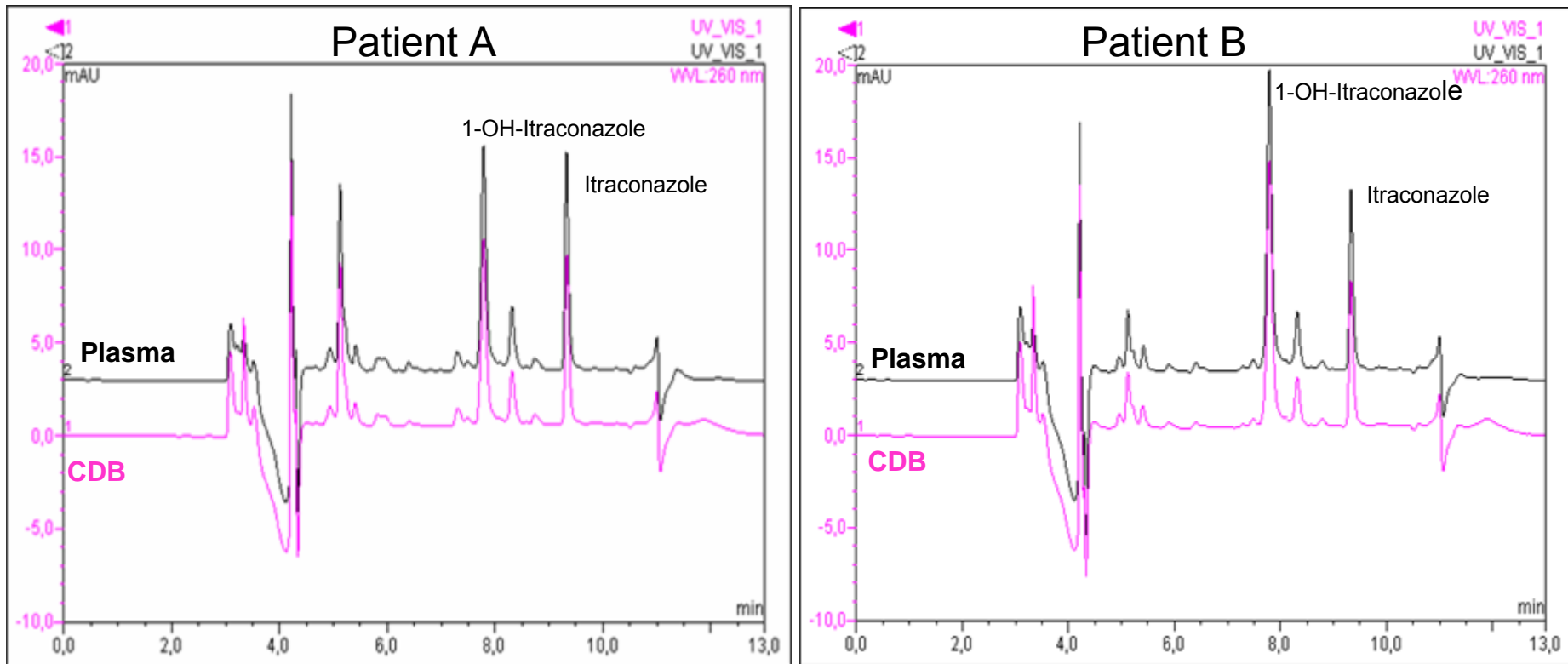
Advantages compared to plasma / serum specimens

- Requires less blood withdrawn
- Enables complete automation / integration of sample pretreatment
- Facilitates traceability of original sample
- Saves time (e.g. no centrifugation / coagulation / labelling)
- Saves consumables (e.g. no secondary tube, pipette tips)
- Reduced risk of infection (completely closed analysis system)
- Enables determination of drug distribution between plasma and cellular components



On-line SPE-LC-UV of Antimycotics :

Comparison of different blood matrices obtained from same patient



SPE-column: LiChrospher® RP4 ADS, 20 x 4 mm ID, dp = 25µm; Flow rate: 2.0 mL/min

Analytical column: Dionex Acclaim® 120 C8, dp = 3µm, 120 Å, 150 x 4.6 mm ID; Flow rate: 1.2 mL/min; 40 °C

Mobile Phase (Fractionation): H₂O/ ACN (98/2, v/v) ; Mobile Phase (Transfer): NH₄AC (0.01M) / ACN (Gradient)

Injection volume: 50 µL



Determination of drug distribution between plasma and red blood cells

$$K_{WB/P} = K_{RBC/P} \times Hct + (1 - Hct)$$

$$C_{CDB} = C_P \times (1 - Hct) + C_{RBC} \times HCT$$

$$C_{RBC} = \frac{C_{CDB} - C_P \times (1 - Hct)}{Hct}$$



$K_{RBC/P}$



Analysis of drugs in CDB :

Advantages compared to plasma / serum specimens

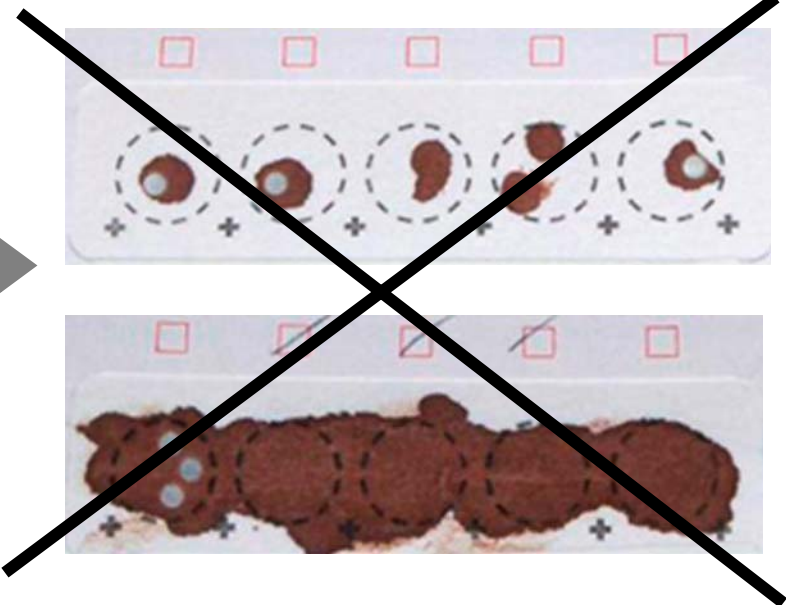
- Requires less blood withdrawn
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- Facilitates traceability of original sample
- Saves time (e.g. no centrifugation / coagulation / labelling)
- Saves consumables (e.g. no secondary tube, pipette tips)
- Reduced risk of infection (completely closed analysis system)
- Enables determination of drug distribution between plasma and cellular components
- Allows analysis of small blood samples (e.g. 10 μL)
 - Pediatrics
 - Point-of-care-testing
 - Phase I studies in small animals



Direct Sampling and Analysis of Capillary Blood



Safety-Lancet
(Sarstedt AG)



CDB is a
unique substitute
for dried blood spots!



Microvette®
(Sarstedt AG)



Sample Vial



Auto-sampler

Preanalytics of CDB : Shipment

Anticoagulated blood and / or CDB samples are shipped according to the international packing instruction P650 for potentially infectious material

A First enclosure : Transport -Blister



DaklaPack Deutschland, Oberhausen, Germany

Preanalytics of CDB : Shipment

B Second enclosure : Safety Bag



DaklaPack Deutschland, Oberhausen, Germany

Preanalytics of CDB : Shipment

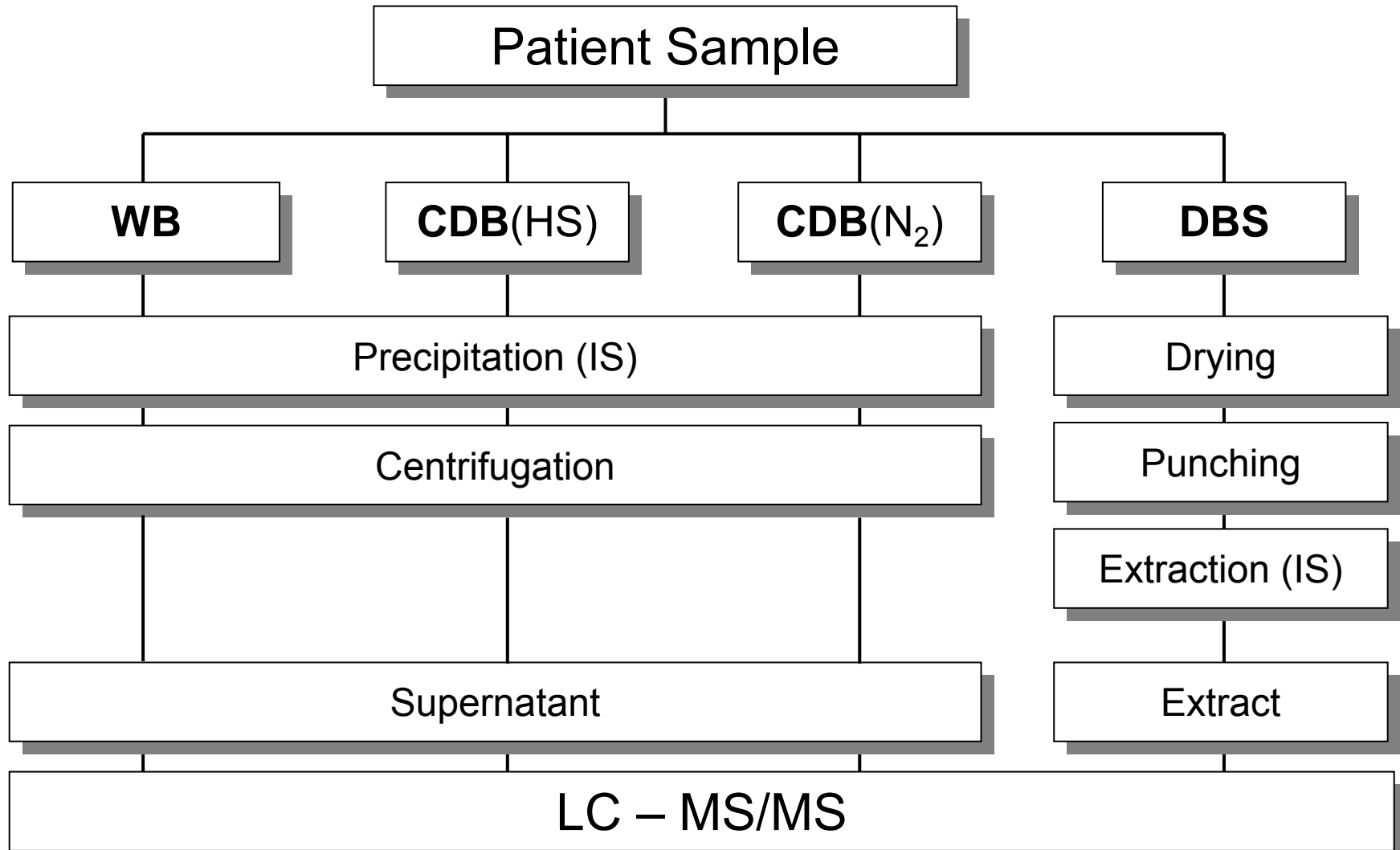
C Final enclosure : Minimailbox



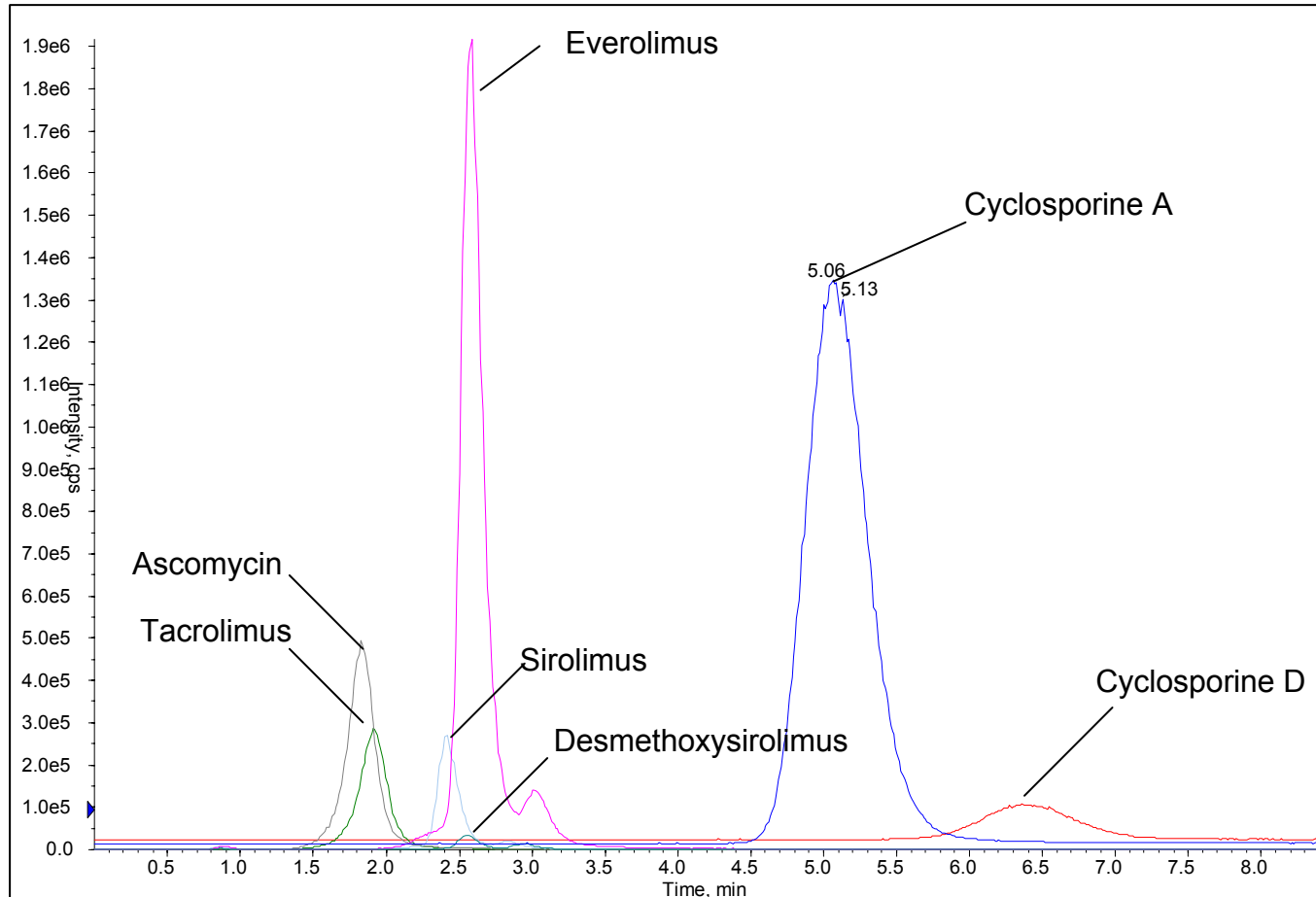
DaklaPack Deutschland, Oberhausen, Germany



Experimental Workflow for Comparison of Methods and Matrices



Standard Chromatogram



- █ Cyclosporine A
- █ Cyclosporine D
- █ Tacrolimus
- █ Ascomycin
- █ Sirolimus
- █ Everolimus
- █ Desmethoxysirolimus

Sample : Solution of immunosuppressants and internal standards, (each 100 ng/mL), MeOH / H₂O (80/20, v/v)

Injection volume : 25 μ L

Separation

POPLC column : C18 (30 mm x 3 mm ID) + C30 (20 mm x 3 mm ID) + CN (30 mm x 3 mm ID) + Phenyl (10 mm x 3 mm ID) , dp 5 μ m

Mobile Phase : MeOH / 2mM NH₄Ac (75/25, v/v) ; Flow-rate : 900 μ L/min ; 8.50 min

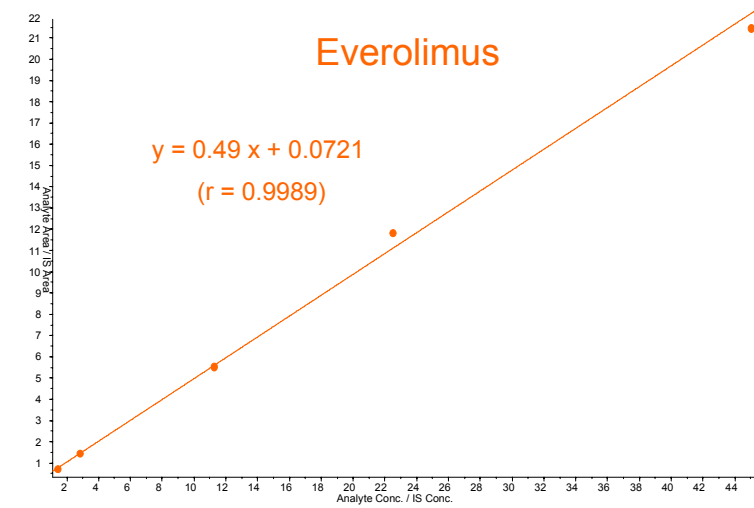
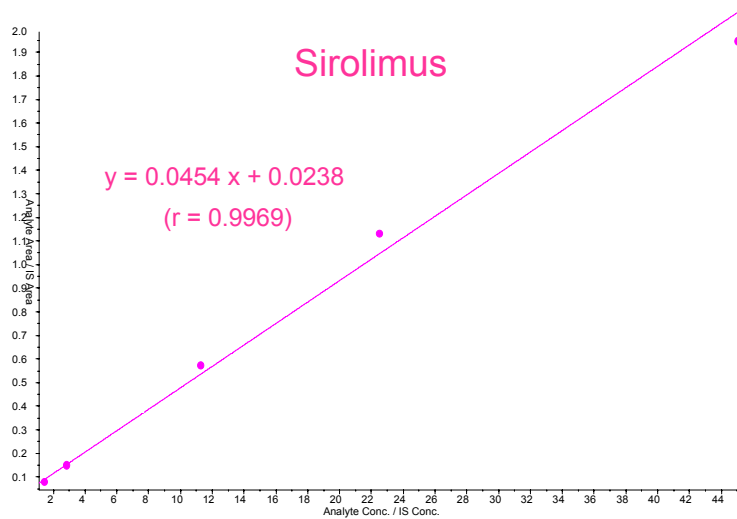
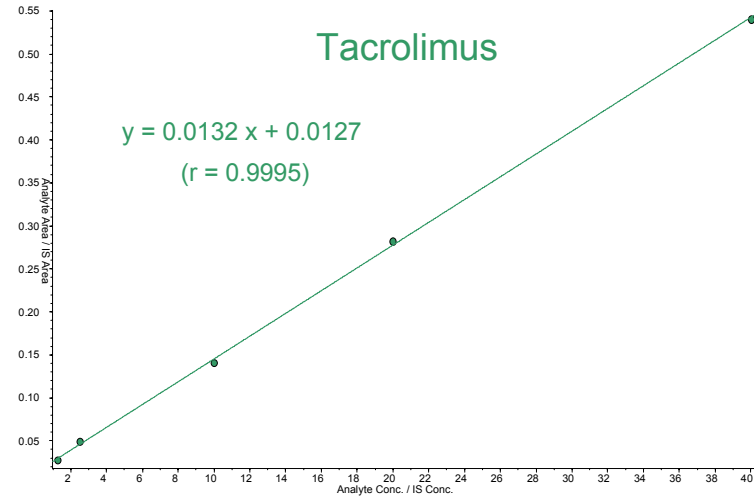
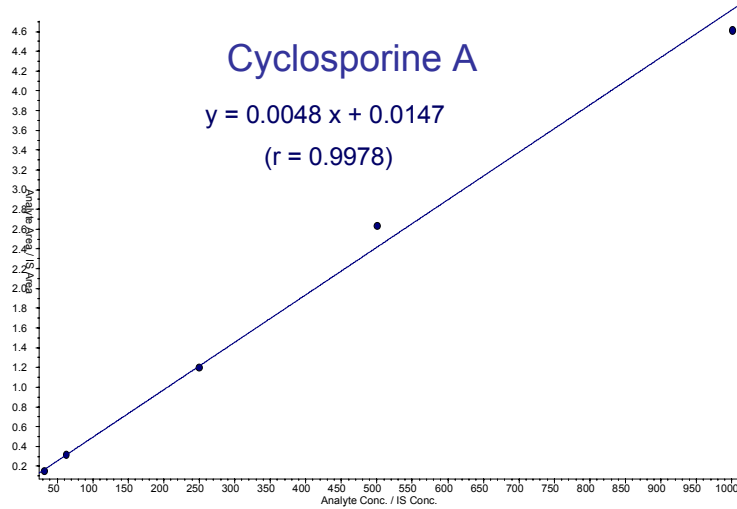
Temperature : 60°C (water bath)

Detection

MDS SCIEX API 5000 LC/MS/MS System with QJet Ion Guide , Applied BioSystems , USA

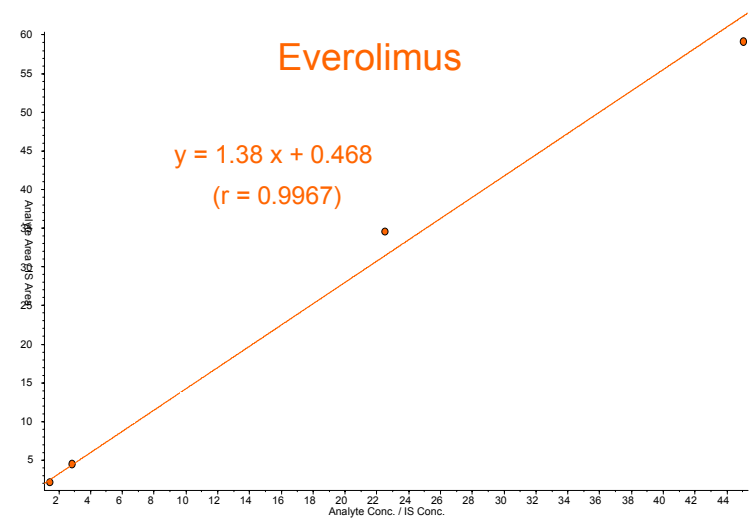
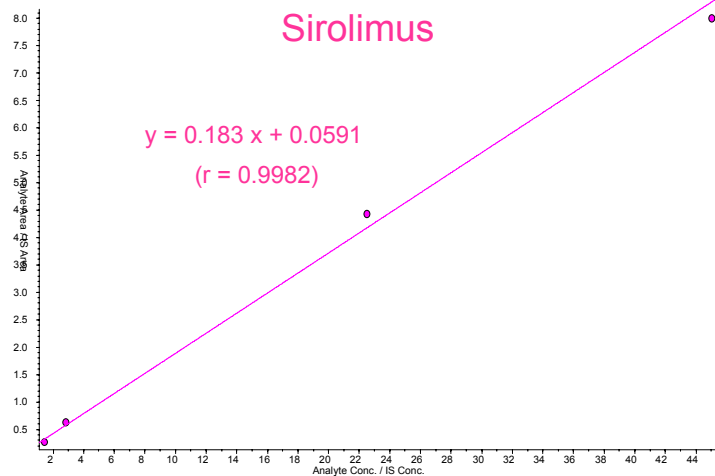
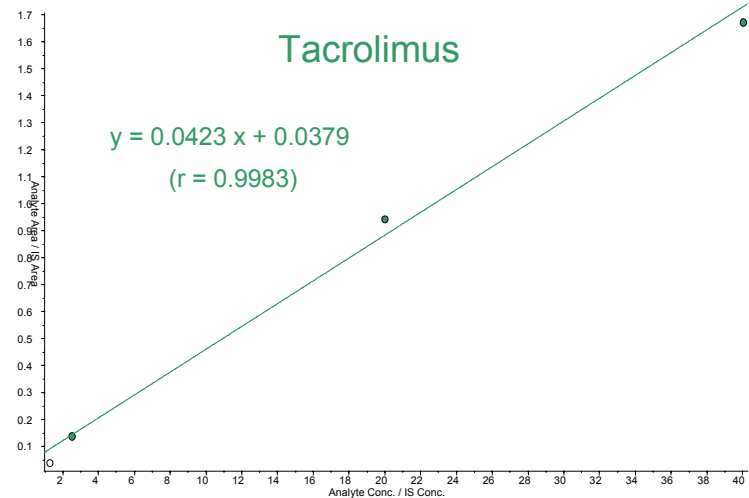
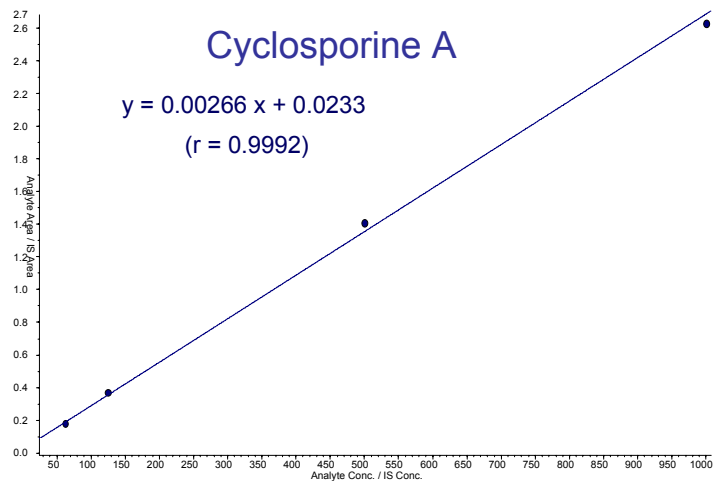
Mode : ESI+

Calibration : WB



Sample preparation : Precipitation of whole blood (spiked with 2.5 vol.% DMSO, containing immunosuppressants with different concentrations)
Injection : 25 μ L aliquot of supernatant

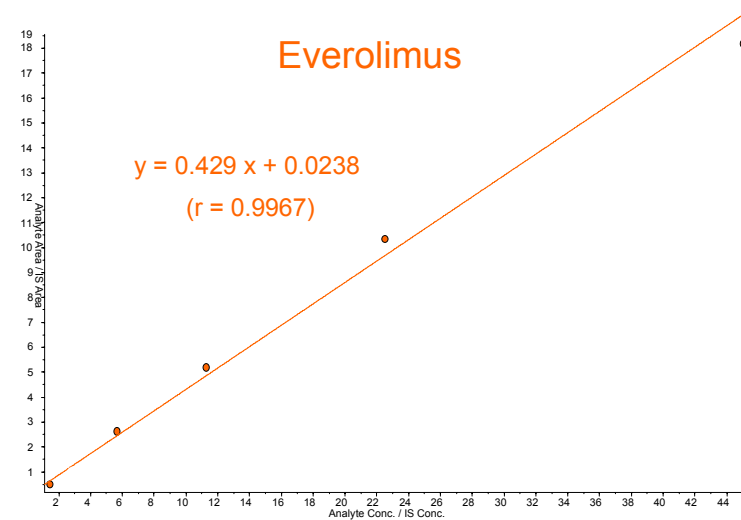
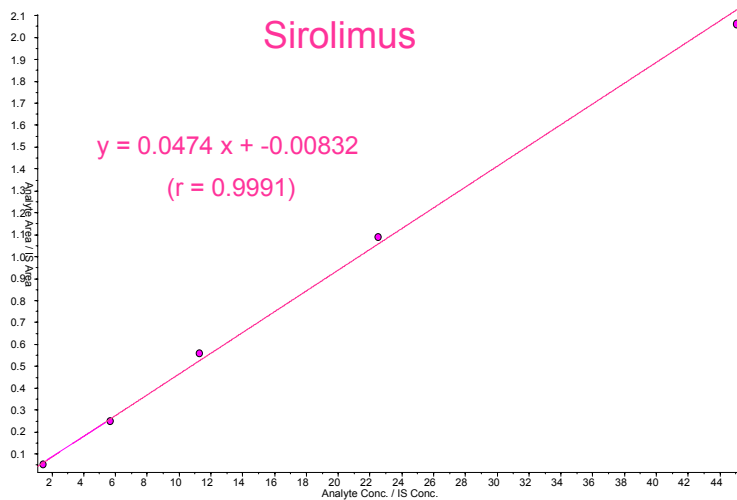
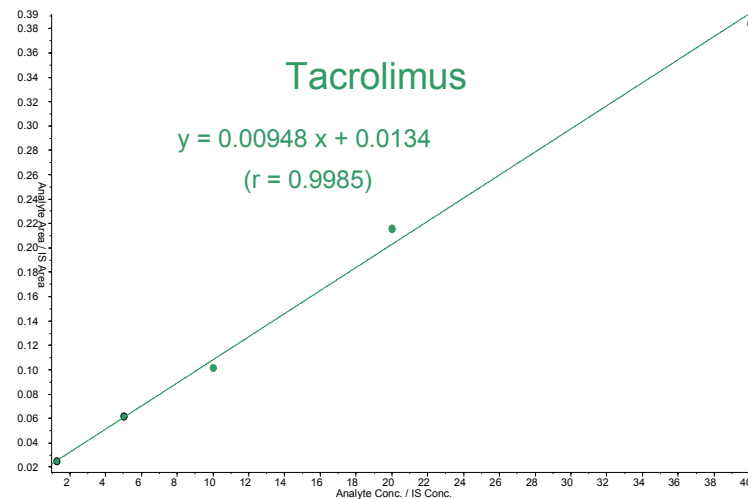
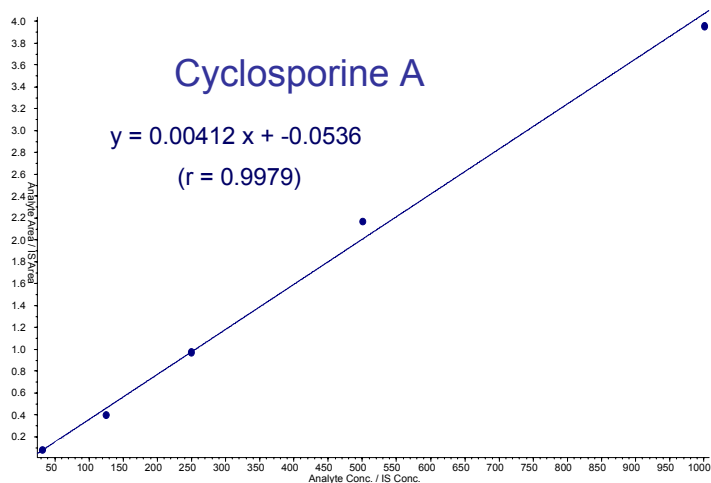
Calibration : DBS



Sample preparation : Extract of DBS (spiked with 2.5 vol.% DMSO, containing immunosuppressants with different concentrations)

Injection : 25 μ L aliquot of supernatant

Calibration : CDB(HS)



Sample preparation : Precipitation of CDB (spiked with 2.5 vol.% DMSO, containing immunosuppressants with different concentrations)

Injection : 25 μ L aliquot of supernatant

Comparison of matrices :

Accuracy and precision (intra-day, n = 5)

	Conc [ng/mL]	WB		DBS		CDB(HS)		CDB(N ₂)	
		Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]
CyA	0.00	-	-						
	31.30	93.1	10.2						
	250.00	93.3	6.0						
	1000.00	106.7	14.6						
Tacro	0,00	-	-						
	1.25	99.1	7.0						
	10.00	93.4	3.6						
	40.00	97.9	6.0						
Siro	0.00	-	-						
	1.41	95.4	8.6						
	11.25	106.6	3.7						
	45.00	97.2	1.2						
Eve	0,00	-	-						
	1.41	83.0	5.6						
	11.25	107.5	3.0						
	45.00	94.1	3.6						

Sample clean-up : Precipitation of WB and CDB
Extraction of DBS

Comparison of matrices :

Accuracy and precision (intra-day, n = 5)

	Conc [ng/mL]	WB		DBS		CDB(HS)		CDB(N ₂)	
		Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]
CyA	0.00	-	-	-	-				
	31.30	93.1	10.2	44.3	31.5				
	250.00	93.3	6.0	63.4	3.1				
	1000.00	106.7	14.6	101.2	4.9				
Tacro	0,00	-	-	-	-				
	1.25	99.1	7.0	65.2	48.3				
	10.00	93.4	3.6	61.5	9.2				
	40.00	97.9	6.0	96.8	4.4				
Siro	0.00	-	-	-	-				
	1.41	95.4	8.6	113.0	16.5				
	11.25	106.6	3.7	70.5	8.2				
	45.00	97.2	1.2	85.5	8.2				
Eve	0,00	-	-	-	-				
	1.41	83.0	5.6	105.6	7.4				
	11.25	107.5	3.0	69.0	8.0				
	45.00	94.1	3.6	88.3	5.8				

Sample clean-up : Precipitation of WB and CDB
Extraction of DBS

Comparison of matrices :

Accuracy and precision (intra-day, n = 5)

	Conc [ng/mL]	WB		DBS		CDB(HS)		CDB(N ₂)	
		Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]
CyA	0.00	-	-	-	-	-	-		
	31.30	93.1	10.2	44.3	31.5	106.8	4.1		
	250.00	93.3	6.0	63.4	3.1	99.7	1.6		
	1000.00	106.7	14.6	101.2	4.9	97.36	0.7		
Tacro	0,00	-	-	-	-	-	-		
	1.25	99.1	7.0	65.2	48.3	99.1	7.8		
	10.00	93.4	3.6	61.5	9.2	93.4	10.1		
	40.00	97.9	6.0	96.8	4.4	97.9	4.7		
Siro	0.00	-	-	-	-	-	-		
	1.41	95.4	8.6	113.0	16.5	95.4	5.2		
	11.25	106.6	3.7	70.5	8.2	106.6	2.2		
	45.00	97.2	1.2	85.5	8.2	97.2	2.3		
Eve	0,00	-	-	-	-	-	-		
	1.41	83.0	5.6	105.6	7.4	83.0	4.3		
	11.25	107.5	3.0	69.0	8.0	107.5	1.1		
	45.00	94.1	3.6	88.3	5.8	94.1	1.8		

Sample clean-up : Precipitation of WB and CDB
Extraction of DBS

Comparison of matrices :

Accuracy and precision (intra-day, n = 5)

	Conc [ng/mL]	WB		DBS		CDB(HS)		CDB(N ₂)	
		Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]	CV [%]
CyA	0.00	-	-	-	-	-	-	-	-
	31.30	93.1	10.2	44.3	31.5	106.8	4.1	100.2	2.7
	250.00	93.3	6.0	63.4	3.1	99.7	1.6	79.3	0.7
	1000.00	106.7	14.6	101.2	4.9	97.36	0.7	95.37	1.8
Tacro	0,00	-	-	-	-	-	-	-	-
	1.25	99.1	7.0	65.2	48.3	99.1	7.8	100.1	3.2
	10.00	93.4	3.6	61.5	9.2	93.4	10.1	91.4	1.1
	40.00	97.9	6.0	96.8	4.4	97.9	4.7	101.5	1.8
Siro	0.00	-	-	-	-	-	-	-	-
	1.41	95.4	8.6	113.0	16.5	95.4	5.2	92.2	4.6
	11.25	106.6	3.7	70.5	8.2	106.6	2.2	106.0	4.3
	45.00	97.2	1.2	85.5	8.2	97.2	2.3	107.2	3.4
Eve	0,00	-	-	-	-	-	-	-	-
	1.41	83.0	5.6	105.6	7.4	83.0	4.3	105.7	2.9
	11.25	107.5	3.0	69.0	8.0	107.5	1.1	106.9	1.6
	45.00	94.1	3.6	88.3	5.8	94.1	1.8	100.2	1.7

Sample clean-up : Precipitation of WB and CDB
Extraction of DBS

CDB(HS) : Inter-day Precision (n = 4 ; [%])

Range	Cyclosporine A	Tacrolimus	Sirolimus	Everolimus
Level 0	-	-	-	-
Level 1	2.0	6.8	7.3	0.5
Level 2	3.2	9.5	6.9	2.2
Level 3	1.8	5.9	2.2	3.6
Level 4	2.1	5.8	4.9	1.7
Level 5	3.8	3.2	3.7	1.3
Level 6	1.7	1.7	3.4	2.5

Level (0 – 6) :

Cyclosporine A (0 ; 31,25 ; 62,5 ; 125 ; 250 ; 500 ; 1000 ng/mL)

Tacrolimus (0 ; 1,25 ; 2,5 ; 5 ; 10 ; 20 ; 40 ng/mL)

Sirolimus (0 ; 1,42 ; 2,82 ; 5,63 ; 11,25 ; 22,5 ; 45 ng/mL)

Everolimus (0 ; 1,42 ; 2,82 ; 5,63 ; 11,25 ; 22,5 ; 45 ng/mL)



Lower Limit of Quantitation [ng/mL]

	WB	DBS	CDB(HS)	CDB(N₂)	Targeted Limit
Cyclosporine A	(8.2)*				30
Tacrolimus	2.6				1
Sirolimus	(0.6)*				1
Everolimus	(0.2)*				1

()*calculated for S/N = 10



Lower Limit of Quantitation [ng/mL]

	WB	DBS	CDB(HS)	CDB(N₂)	Targeted Limit
Cyclosporine A	(8.2)*	60.1			30
Tacrolimus	2.6	9.5			1
Sirolimus	(0.6)*	4.3			1
Everolimus	(0.2)*	1.63			1

()*calculated for S/N = 10



Lower Limit of Quantitation [ng/mL]

	WB	DBS	CDB(HS)	CDB(N₂)	Targeted Limit
Cyclosporine A	(8.2)*	60.1	(12.4)*		30
Tacrolimus	2.6	9.5	2.5		1
Sirolimus	(0.6)*	4.3	(1.3)*		1
Everolimus	(0.2)*	1.63	(0.2)*		1

()*calculated for S/N = 10



Lower Limit of Quantitation [ng/mL]

	WB	DBS	CDB(HS)	CDB(N₂)	Targeted Limit
Cyclosporine A	(8.2)*	60.1	(12.4)*	(3.1)*	30
Tacrolimus	2.6	9.5	2.5	(1.0)*	1
Sirolimus	(0.6)*	4.3	(1.3)*	(0.5)*	1
Everolimus	(0.2)*	1.63	(0.2)*	(0.1)*	1

()*calculated for S/N = 10



Recovery of Immunosuppressants from DBS

	Concentration [ng/mL]	Recovery DBS* n = 2 [%]	CV n = 2 [%]		Concentration [ng/mL]	Recovery DBS* n = 2 [%]	CV n = 2 [%]
Cyclosporine A	31,25	< LLOQ	6,6	Sirolimus	1,25	< LLOQ	2,6
	62,50				2,81		
	125,00				5,63		
	250,00	61,63	10,25		98,44		
	500,00	113,9**	-		22,50		
	1000,00	88,52	1,8		45,00		
Tacrolimus	1,25	< LLOQ	2,6	Everolimus	1,25	< LLOQ	11,8
	2,50				2,81		
	5,00				5,63		
	10,00	77,34	10,25		111,54		
	20,00	119,38	4,5		22,50	98,47	
	40,00	101,77	14,0		45,00	0,7	

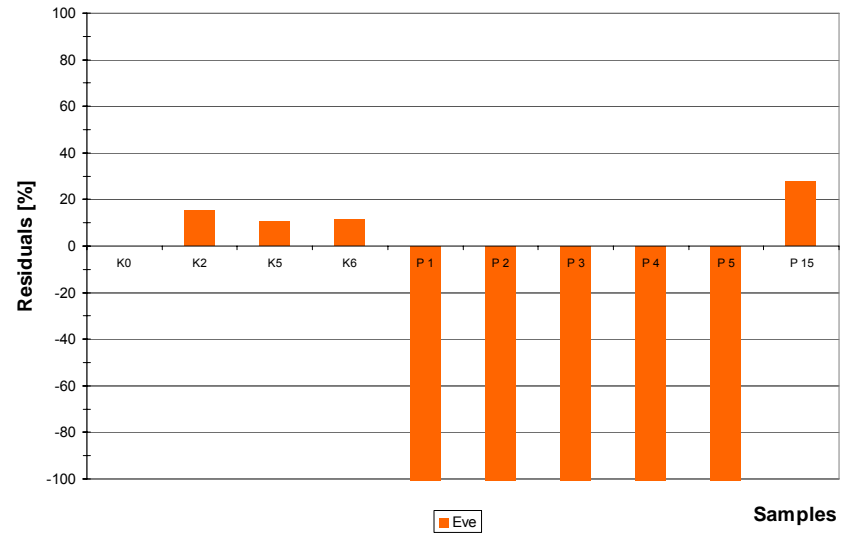
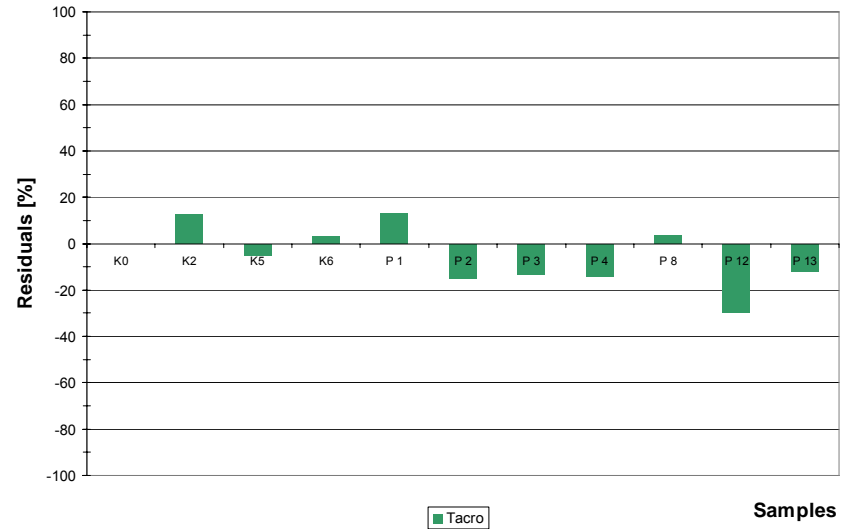
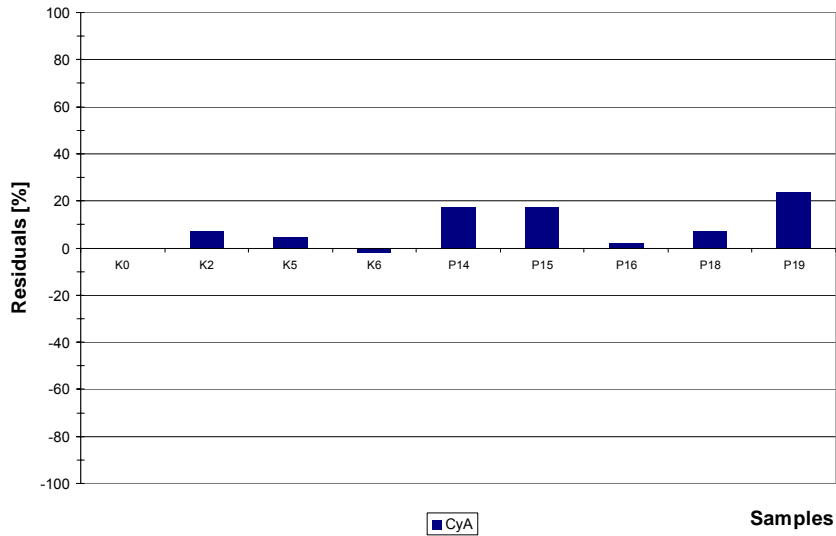
* mixture of standard solution = 100%, spotted 3x10µL

** n = 1

LLOQ = lower limit of quantification



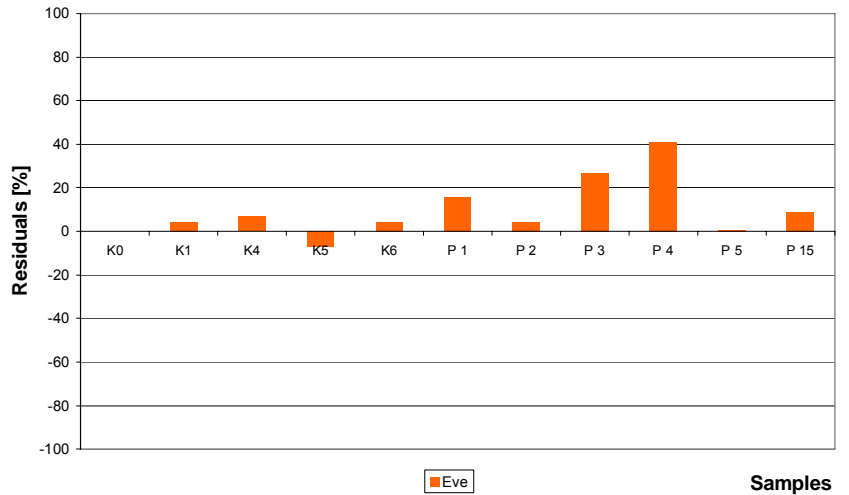
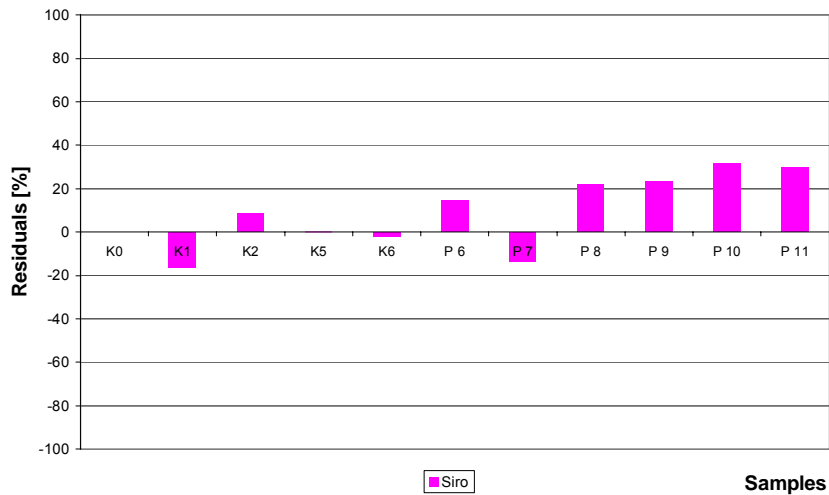
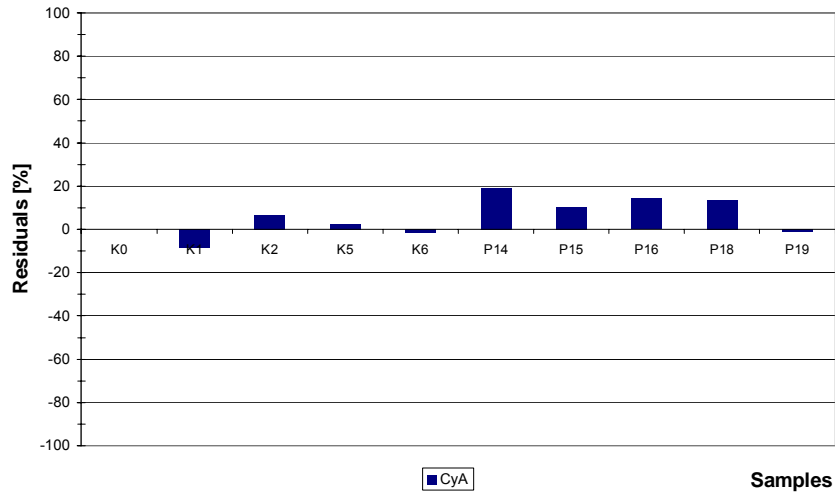
Comparison of Matrices [Residuals Plot] : WB *versus* DBS



K0 – K6 = Calibrators; P = Patient samples; n = 1



Comparison of Matrices [Residuals Plot] : WB *versus* CDB(N₂)



K0 – K6 = Calibrators; P = Patient samples; n = 1

Analysis of drugs in Cell-Disintegrated Blood (CDB) : Advantages compared to Dried Blood Spots (DBS)

- Sampling does not require a defined volume (pipetting step)
- Sampling is faster as no drying process is involved
- Sampling device (vial) is much cheaper than a filter card
- Internal standard is added to original sample and not to extraction solvent
- Better accuracy and precision
- Hyphenation with fully automated clean-up techniques, e.g. on-line SPE
- Reduced risk of infection (completely closed analysis system)
- Analyses of larger sample size, e.g. 50 μL .
(30 μL of blood yield a spot of $r = 4$ mm on a typical filter card. A disc with $r = 1.5$ mm contains 4 μL of blood.)
- No dependency on hematocrit
- Facilitates traceability of original sample (“Vial-to-File”)
- Enables determination of drug distribution between plasma and cellular components



Outlook : Automated sampling of blood in living animals followed by in-line processing and on-line SPE-LC-MS/MS analysis



Animal(s) → AccuSampler[®] → or → On-line SPE → LC-MS/MS

BloodLyser[®]
or
BloodCler[®]

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LEAP Technologies

Thank you !

