Supplemental Digital Content 1, containing 4 Figures and 2 Tables; also see the Supplemental Digital Content 2. The Supplemental Digital Content was not copyedited by *Archives of Pathology & Laboratory Medicine*.

APPLICATION OF A FULLY AUTOMATED DRIED BLOOD SPOT METHOD FOR THERAPEUTIC DRUG MONITORING OF IMMUNOSUPPRESSANTS: ANOTHER STEP TOWARDS IMPLEMENTATION OF DRIED BLOOD SPOT ANALYSIS

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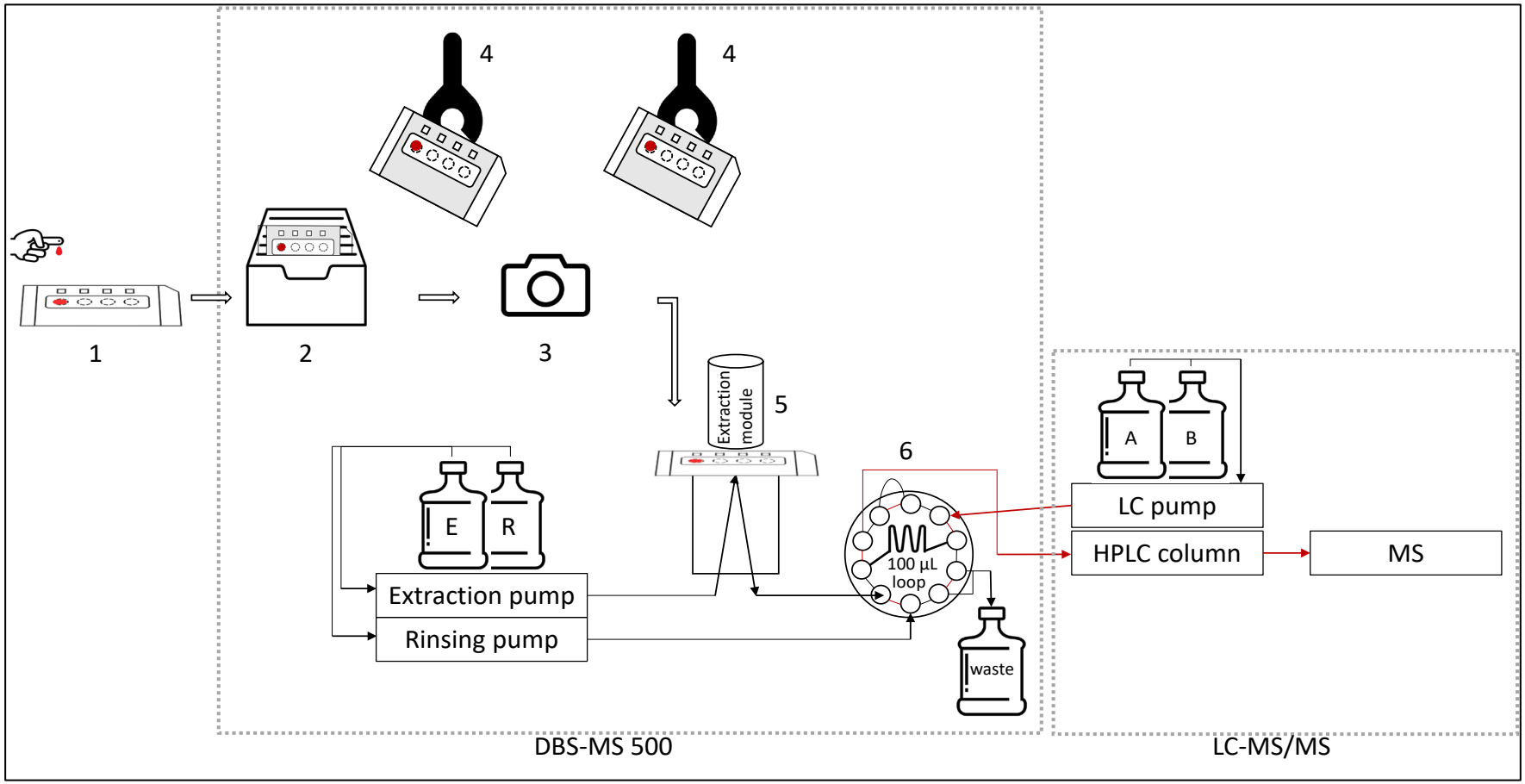
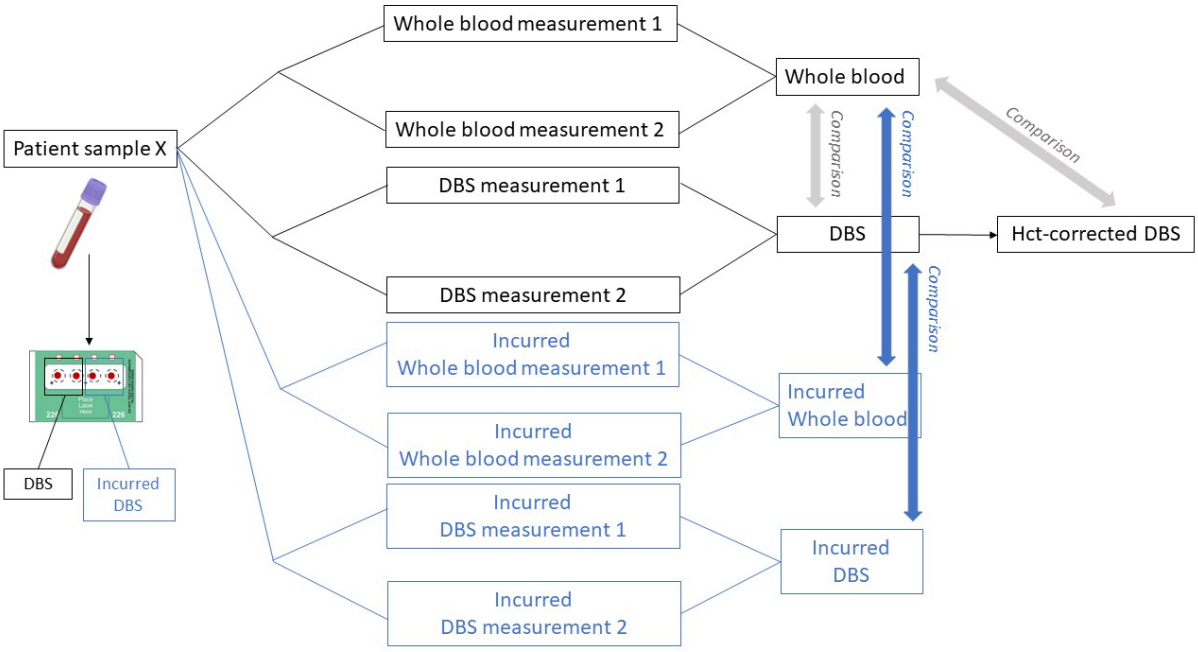
Supplemental Figure 1



Supplemental Figure 1. Fully automated DBS-MS 500 workflow.
[1] Sample collection; [2] Dried Blood Spot (DBS) card rack; [3] built-in camera; [4] robotic gripper; [5] extraction module containing a 4 mm clamp head; [6] 10-port valve equipped with a 100 µL sample loop; [E] extraction solvent: methanol/water (80/20, v/v) containing the Internal Standard (IS) mixture); [R] rinsing solvent: methanol/water/2-propanol/acetonitrile (25/25/25/25, v/v/v/v/); [A] mobile phase A: 10 mM ammonium formate in water containing 0.1% Formic Acid (FA); and [B] mobile phase B: acetonitrile/water (90/10, v/v) containing 10 mM ammonium formate and 0.1% FA; [LC] liquid chromatography pump; [HPLC] high-pressure liquid chromatography column: Kinetex 2.6 µm Phenylhexyl 50 x 2.1 mm column; [MS] Mass Spectrometer.

Supplemental Figure 2



Supplemental Figure 2. Overview of the different Dried Blood Spot (DBS) and whole blood analyses performed and comparisons made in the manuscript.

Supplemental Figure 3

$$y = ax + b \quad (1)$$

With $x = hct$

$$y = \frac{(DBS-WB)}{WB} * 100$$

$$\frac{(DBS - WB)}{WB} * 100 = a * hct + b \quad (2)$$

$$\left(\frac{DBS}{WB} - \frac{WB}{WB}\right) * 100 = a * hct + b \quad (3)$$

$$\left(\frac{DBS}{WB} - 1\right) * 100 = a * hct + b \quad (4)$$

$$\frac{DBS}{WB} = a * hct + b + 1 \quad (5)$$

$$\frac{DBS}{(a * hct) + b + 1} = WB \quad (6)$$

$With\ a = -1.559$

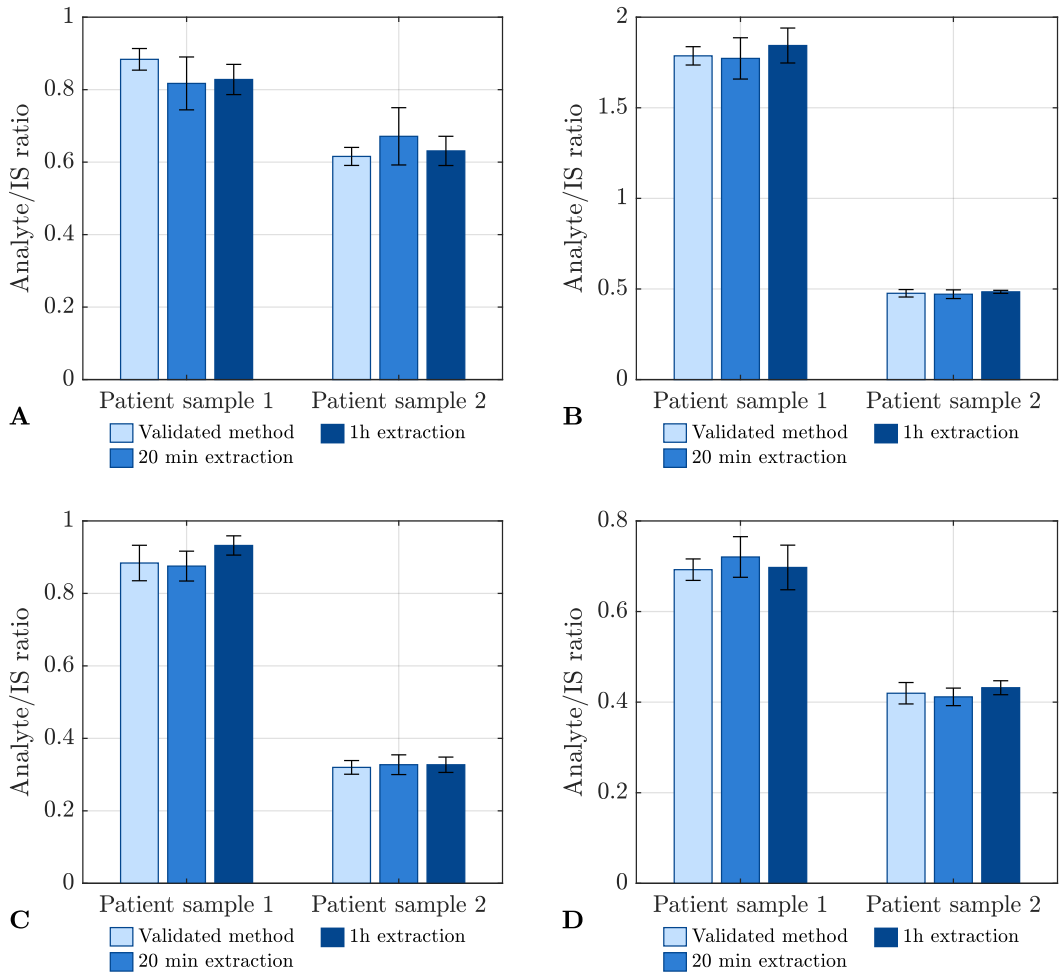
$b = 0.6305$

$WB = DBS_{corrected}$

$$\frac{DBS}{-1.559 * hct + 1.6305} = DBS_{corrected} \quad (7)$$

Supplemental Figure 3. Formula final hematocrit (hct) correction with mean of duplicate Dried Blood Spot measurements (DBS) and mean of duplicate whole blood measurements (WB). (1) Equation of a linear regression analysis with slope a and intercept b. (2) y equals the ratio of the difference between the DBS and WB concentration divided by the WB concentration in percentage. (3), (4) and (5) Describe a simplification of this formula. (6) Since we are interested in the relationship between DBS and WB, the formula is reduced in function of the WB concentration. Additionally, the a and b values, as found in the manuscript are mentioned. (7) The measured DBS concentration (here 'DBS') is needed to calculate an adapted DBS value based on a, b and hct. Therefore, WB is substituted by '$DBS_{corrected}$' in the final formula.

Supplemental Figure 4



Supplemental Figure 4. Analyte:IS ratio in function of extraction time for tacrolimus (A), sirolimus (B), everolimus (C) and cyclosporin A (D) for two different patient samples per analyte (n = 5). 'Validated method' indicates the validated method according to Deprez and Stove, 2021 in Journal of Chromatography A.

Supplemental Table 1. *Results incurred sample reanalysis for each analyte in whole blood and Dried Blood Spots (DBS), quantified against a frozen and for DBS also against a fresh calibration curve.*

		Incurred sample reanalysis (frozen curve)			Incurred sample reanalysis (fresh curve)		
Analyte	**Matrix**	**Number of samples**	**% of the samples meeting acceptance criterion [absolute numbers]**		**Number of samples**	**% of the samples meeting acceptance criterion [absolute numbers]**	
Tacrolimus	**DBS**	39	97%	[38/39]	39	100%	[39/39]
	Whole blood	39	95%	[37/39]		*No Data*	
Sirolimus	**DBS**	45	93%	[42/45]	45	87%	[39/45]
	Whole blood	45	98%	[44/45]		*No Data*	
Everolimus	**DBS**	43	98%	[42/43]	43	91%	[39/43]
	Whole blood	42	98%	[41/42]		*No data*	
Cyclosporin A	**DBS**	56[a]	100%	[56/56]	56	84%	[47/56][c]
	Whole blood	55[b]	98%	[54/55]		*No data*	

[a] one outlier was excluded from data analysis via Grubbs test and two samples were below the lower limit of quantification (LLOQ), [b] one sample below LLOQ. [c] The worse agreement using the fresh curve compared to the frozen curve can be found in a single batch of samples where all samples were deviating compared to the first analysis, possibly due to calibration differences. We did not find an objective reason to exclude that batch.

Supplemental Table 2. *Accuracy (%bias) and within-day (repeatability) and total precision (%CV) for QCs of tacrolimus, sirolimus, everolimus, cyclosporin A at four concentration levels in whole blood (WB) and DBS samples (DBS) (n = 5 × 2).*

	WB			DBS		
QC sample	**Accuracy (bias)**	**Repeatability (CV)**	**Total imprecision (CV)**	**Accuracy (bias)**	**Repeatability (CV)**	**Total imprecision (CV)**
			Tacrolimus			
LLOQ	3.0%	5.0%	9.2%	-3.7%	7.9%	8.9%
QCL	-3.3%	3.7%	6.7%	-3.6%	9.3%	11.4%
QCM	-1.8%	7.1%	8.0%	-4.9%	6.7%	9.8%
QCH	1.8%	7.8%	9.7%	-1.1%	8.9%	8.9%
			Sirolimus			
LLOQ	-7.6%	10.1%	16.8%	3.0%	9.5%	19.1%
QCL	-5.3%	8.5%	9.0%	-1.4%	12.5%	16.1%
QCM	-7.5%	3.4%	3.4%	-1.8%	7.6%	14.0%
QCH	-6.1%	4.2%	8.5%	-0.1%	7.2%	13.4%
			Everolimus			
LLOQ	-15.6%	9.9%	11.2%	-20.0%	9.6%	16.2%
QCL	-10.9%	6.0%	8.9%	1.8%	9.3%	9.8%
QCM	-8.2%	7.2%	7.8%	-2.4%	7.8%	7.8%
QCH	-2.4%	8.5%	8.5%	-6.5%	8.8%	10.2%
			Cyclosporin A			
LLOQ	-10.5%	2.9%	7.5%	-10.4%	4.6%	10.2%
QCL	-2.3%	3.3%	6.2%	3.3%	5.9%	5.9%
QCM	-5.1%	8.7%	8.7%	-6.3%	2.6%	3.9%
QCH	-3.0%	3.3%	4.7%	-2.8%	2.7%	5.7%

Abbreviations: Quality Control (QC), lower limit of quantification (LLOQ), Low Quality Control (QCL), Medium Quality Control (QCM), High Quality Control (QCH). Data as determined in the method validation (Deprez S, Stove CP. Fully Automated Dried Blood Spot Extraction coupled to Liquid Chromatography-tandem Mass Spectrometry for Therapeutic Drug Monitoring of Immunosuppressants. J Chromatogr A. 2021;1653. doi:10.1016/j.chroma.2021.462430).