Journal of Visualized Experiments

Quantification of the Immunosuppressant Tacrolimus on Dried Blood Spots Using LC-MS/MS

--Manuscript Draft--

Manuscript Number:	JoVE52424R3
Full Title:	Quantification of the Immunosuppressant Tacrolimus on Dried Blood Spots Using LC-MS/MS
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Tacrolimus, dried blood spots, high-performance liquid chromatography, tandem mass spectrometry, LC-MS/MS, column switching, online extraction, therapeutic drug monitoring, home monitoring, adherence monitoring
Manuscript Classifications:	5.1: Diagnosis
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Abstract:	Here we describe a high-performance liquid chromatography- tandem mass spectrometry (HPLC-MS/MS) assay to quantify the immunosuppressant tacrolimus in dried blood spots using a simple manual protein precipitation step and online column extraction. The calcineurin inhibitor tacrolimus is the cornerstone of most immunosuppressive treatment protocols after solid organ transplantation in the United States. Tacrolimus is a narrow therapeutic index drug and as such requires therapeutic drug monitoring and dose adjustment based on its whole blood trough concentrations. To facilitate home therapeutic drug and adherence monitoring, the collection of dried blood spots is an attractive concept. After a finger stick, the patient collects a blood drop on filter paper at home. After the blood is dried, it is mailed to the analytical laboratory. For tacrolimus analysis a ¼" disc is punched from the center of the blood spot. The blood spot is homogenized using a bullet blender and then proteins are precipitated with methanol/ 0.2 M ZnSO4 containing the internal standard D2,13C-tacrolimus. After vortexing and centrifugation, 100 µl supernatant is injected onto an online extraction column and washed with 5 ml/min of 0.1 formic acid/ acetonitrile (7:3, v:v) for 1 min. Hereafter, the switching valve is activated and the analytes are back-flushed onto the analytical column (and separated using a 0.1% formic acid/ acetonitrile gradient. Tacrolimus is quantified in the positive multi reaction mode (MRM) using a tandem

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Author Comments:	
Additional Information:	
Question	**Response**
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Short Abstract:
Here we describe a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay to quantify the immunosuppressant tacrolimus in dried blood spots using a simple manual protein precipitation step and online column extraction.

Long Abstract:
The calcineurin inhibitor tacrolimus is the cornerstone of most immunosuppressive treatment protocols after solid organ transplantation in the United States. Tacrolimus is a narrow therapeutic index drug and as such requires therapeutic drug monitoring and dose adjustment based on its whole blood trough concentrations. To facilitate home therapeutic drug and adherence monitoring, the collection of dried blood spots is an attractive concept. After a finger stick, the patient collects a blood drop on filter paper at home. After the blood is dried, it is mailed to the analytical laboratory where tacrolimus is quantified using high-performance liquid chromatography- tandem mass spectrometry (HPLC-MS/MS) in combination with a simple manual protein precipitation step and online column extraction.

For tacrolimus analysis, a 6-mm disc is punched from the saturated center of the blood spot. The blood spot is homogenized using a bullet blender and then proteins are precipitated with methanol/ 0.2 M $ZnSO_4$ containing the internal standard $D_{2,}{}^{13}C$-tacrolimus. After vortexing and centrifugation, 100 µL of supernatant is injected into an online extraction column and washed with 5 mL/min of 0.1 formic acid/ acetonitrile (7:3, v:v) for 1 min. Hereafter, the switching valve is activated and the analytes are back-flushed onto the analytical column (and separated using a 0.1% formic acid/ acetonitrile gradient). Tacrolimus is quantified in the positive multi reaction mode (MRM) using a tandem mass spectrometer.

The assay is linear from 1- 50 ng/mL. Inter-assay variability (3.6- 6.1%) and accuracy (91.7-101.6%) as assessed over 20 days meet acceptance criteria. Average extraction recovery is 95.5%. There are no relevant carry-over, matrix interferences and matrix effects. Tacrolimus is stable in dried blood spots at room temperature and at +4 °C for 1 week. Extracted samples in the autosampler are stable at +4 °C for at least 72 h.

Introduction:
Tacrolimus is a potent immonosuppressant[1-7] that has a macrolide structure[8] (Figure 1). Due to cis-trans isomerism of the C-N bonds it forms two rotamers in solution[9] that can be separated by reversed phase high-performance liquid chromatography (HPLC) Tacrolimus is lipophilic and soluble in alcohols (methanol: 653 g/L, ethanol: 355 g/L),

halogenated hydrocarbons (chloroform: 573 g/L) and ether. It is sparingly soluble in aliphatic hydrocarbons (hexane: 0.1 g/L and water (pH 3: 0.0047 g/L)[9]. The molecule does not contain any chromophore and its UV-absorption maximum is 192 nm. Tacrolimus acts *via* inhibition of calcineurin. Its mechanism of action has been reviewed in references[10,11]. It is currently used in more than 80% of solid-organ transplant patients in the United States[12].

The therapeutic index of tacrolimus is considered to be narrow[13]. In addition, the correlation between tacrolimus doses and blood concentrations is poor and pharmacokinetics is variable[14,15]. Therapeutic drug monitoring to guide tacrolimus dosing in transplant patients is therefore general clinical practice[16-20]. The goal is to keep the tacrolimus blood concentrations within a pre-defined therapeutic range. Tacrolimus blood concentrations below the therapeutic range may result in increased activity of chronic or acute allo-immune reactions, while concentrations above the therapeutic window increase the risk for over-immunosuppression, cancer and toxicities, such as nephrotoxicity, neurotoxicity, hypertension, and diabetes. High pharmacokinetic intra-individual variability of tacrolimus may be detrimental to both transplant organ and patient survival[21,22]. While inter-individual variability of tacrolimus pharmacokinetics is mainly caused by CYP3A5 polymorphisms, reasons for intra-individual variability include, but are not limited to, drug-drug, disease-drug and food-drug interactions[14,15]. Also lack of adherence to the immunosuppressive therapeutic drug regimen is a contributing factor and a major reason for graft loss[23,24].

These considerations suggest that frequent home therapeutic drug and adherence monitoring of tacrolimus whole blood concentrations may be beneficial to ensure that patients have tacrolimus exposure within the desired therapeutic window at all times. However, the logistics and cost of more frequent therapeutic drug monitoring as it is current clinical practice[15] is prohibitive. One of the reasons is that the patient has to see a phlebotomist to have the required venous blood sample drawn. Dried blood spots have recently emerged as an attractive concept[25-28]. After a simple finger stick the patient collects a blood drop on a special filter paper card and after the blood spot has dried, it can be mailed to a central laboratory for analysis of tacrolimus and any other immunosuppressant that the patient may currently be taking. This has become possible due to the development of highly sensitive and specific LC-MS/MS assays for the quantification of tacrolimus and other immunosuppressants in very small blood volumes such as dried blood spots (typically 20 µL of blood)[25,29-43]. Another advantage is that minimally invasive, low volume sample collection strategies such as dried blood spots greatly facilitate therapeutic drug monitoring and pharmacokinetic studies in small children[28].

Tacrolimus is usually measured in venous EDTA whole blood[15]. Reasons are that tacrolimus extensively distributes into blood cells and that clinical studies have reported better correlation between tacrolimus trough concentrations in blood than in plasma with clinical events[15,18]. In comparison, the analysis of tacrolimus in dried blood spots is based on capillary blood that is mixed with the filter paper matrix. This presents challenges in terms of solubilization of tacrolimus and potential interferences

with the LC-MS/MS analysis. Here we present an established and validated assay based on homogenization of the dried blood spot using a bullet blender in combination with a high-flow online column sample clean up procedure and LC-MS/MS analysis. As of today, this assay has successfully been used for the quantification of more than five thousand tacrolimus dried blood spot samples for adherence monitoring in clinical trials.

Protocol:

De-identified blood samples from healthy individuals were from the University of Colorado Hospital (Aurora, Colorado). The use of de-identified blood bank samples for validation studies as well as for the preparation of calibrators and quality control samples was considered “exempt” by the Colorado Multi-institutional Review Board (COMIRB, Aurora, Colorado).

1. Preparation of references and solutions

1.1. Purchase tacrolimus and the internal standard $D_{2,}{}^{13}C$-tacrolimus from the vendors listed in the Table of Materials and Equipment.

1.1.1. Prepare stock solutions in pure methanol at a concentration of 1 mg/mL for tacrolimus and a concentration of 10 µg/mL for $D_{2,}{}^{13}C$-tacrolimus. Make stock solutions of reference materials based on three independent weightings. Aliquot stock solutions and store at -70 °C or below.

1.2. Prepare solution to precipitate proteins and extract tacrolimus using methanol / 0.2 M $ZnSO_4$ in water (7:3, v:v). This solution also contains the internal standard $D_{2,}{}^{13}C$-tacrolimus at a concentration of 2.5 ng/mL and is used for the extraction of all samples except for the extraction of blank samples (please see 1.3.3.).

1.2.1. Prepare this protein precipitation solution freshly on each extraction day and set the expiration of the solution at 12 h.

1.3. Preparation of calibration curve and quality control (QC) samples

1.3.1. Prepare stock solutions of tacrolimus by performing appropriate dilutions of the stock solution using pure methanol.

1.3.2. To prepare calibrators and quality control samples, spike 20 µL of appropriately diluted stock solution into EDTA whole blood, incubate at 37 °C under gentle shaking in a water bath to allow for homogeneous distribution of tacrolimus into the cellular blood components for 20 min and aliquot into 1.5 mL polypropylene tubes with conical bottom and snap-on lids. Ensure that the relative volume of organic solvent does not exceed 5%.

1.3.3. Spot 50 µL of the spiked whole blood into the middle of each circle on the filter cards using a pipette.

1.3.4. Dry the blood spots on filter cards at room temperature for 3 h.

1.3.5. Prepare tacrolimus calibration standards in human EDTA whole blood at tacrolimus concentrations of 1, 2.5, 5, 10, 25, and 50 ng/mL. Prepare a blank sample for extraction like the calibration standards with the protein precipitation solution containing the internal standard $D_{2,}{}^{13}C$-tacrolimus ("zero sample").

1.3.6. Prepare QC samples in human EDTA whole blood at concentrations of 0, 2, 4, 20, 40 ng/mL. Prepare a blank sample. In contrast to the QC samples that are extracted with precipitation containing the internal standard $D_{2,}{}^{13}C$-tacrolimus, extract this blank sample with protein precipitation solution that does not contain the internal standard $D_{2,}{}^{13}C$-tacrolimus ("blank sample").

1.4. Collection of clinical samples

1.4.1. Collect dried blood spots as described in[43,44].

2. Extraction of tacrolimus dried blood spot samples.

2.1. Visually inspect the dried blood spot to ensure acceptable sample quality and volume[45].

2.2. Punch center of the blood spot on the filter card with a 6-mm hole punch.

Note: The quality of punches may be monitored by weighing. A punched saturated filter disc weighs in average 5.02 mg ± 0.09 mg (range: 4.83- 5.14 mg, n=12).

2.3. Place discs into 1.5 mL polypropylene tubes with conical bottom and snap-on lids.

2.4. Add 20-30 bullets to each tube.

2.5. Add 500 µL of the protein precipitation solution (methanol: 0.2 M $ZnSO_4$, 7:3, v:v with 2.5 ng/mL internal standard) into each tube. For the extraction of blank samples, use protein precipitation solution without the internal standard.

2.6. Homogenize the discs in the bullet blender for 1 min (maximum speed, setting "10").

2.7. Shake samples at room temperature on multi-tube vortex (maximum speed, setting "10") for 10 min.

2.8. Centrifuge samples at 16.000 x g and 4 °C for 10 min.

2.9. Transfer the supernatants into glass HPLC vials equipped with a 300 µL insert. Use pre-slit Teflon seals.

Note: Extracted samples may be stored at -20 °C or below until LC-MS/MS analysis.

3. LC-MS/MS analysis

3.1. Load 100 µL of the supernatant of the extracted sample onto the C8 cartridge extraction column and wash with a 7:3 ratio of 0.1% formic acid in water: acetonitrile at a flow of 5 mL/min for 1 min. The connections of the switching valve are shown in Figure 2 and the gradient run by the extraction pump in Table 1.

3.2. Hereafter, activate the switching valve resulting in back-flush of the analytes from the pre-column onto the analytical column.

3.3. Set the column thermostat to 65 °C.

3.4. Elute the analytes from the analytical column using the flow rates and gradient shown in Table 1.

3.5. Connect the analytical column to a tandem mass spectrometer *via* the turbo electrospray ionization source. Adjust the key parameters of the mass spectrometer according to Table 2.

3.6. Detect positive ions ([M+Na]+) in the multiple reaction mode (MRM). Use the following ion transitions for quantification: tacrolimus: m/z (mass/charge) = 826.6 → 616.2 and D_2,^{13}C-tacrolimus: m/z= 829.6 → 619.2.

Note: The total run time is 4.6 min.

4. Quantification

4.1. For each run, generate a calibration curve based on the calibrators prepared in 1.3.5 and include in each analytical run.

4.1.1. Generate a calibration curve by plotting nominal concentrations *versus* response factor of analyte (Peak Area [Analyte] / Peak Area [internal standard]) using the mass spectrometer software.

4.1.2. Fit the calibrators using a quadratic fit in combination with 1/X weighting.

4.2. To quantity tacrolimus in the dried blood spots integrate the tacrolimus and internal standard peaks in the extracted MRM chromatograms. Calculate the response factor for tacrolimus (Peak Area [Analyte] / Peak Area [internal standard]) and compare with the calibration curve using the mass spectrometry software.

5. Validation procedures

5.1. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ).

5.1.1. Consider the lowest tacrolimus concentration with a peak-to-noise ratio of 4:1 as the lower limit of detection (LLOD). Define the lower limit of quantification (LLOQ) as the lowest concentration of the calibration curve with an accuracy equal to or better than ± 20% deviation from the nominal concentration and precision equal to or better than 20% (coefficient of variance).

5.2. Intra- and inter-day accuracies and precisions.

5.2.1. Test the accuracy and precision at four concentration levels of 2 ng/mL (QC1), 4 ng/mL (QC2), 20 ng/mL (QC3) and 40 ng/mL (QC4).

5.2.2. Prepare the QC samples on each validation day in human EDTA whole blood, dry on filter cards, extract, and analyze as described above.

5.2.3. Determine intra-day accuracy and precision with 6 samples per QC concentration level.

5.2.4. Assess inter-day accuracy and precision over 20 days. Measure each QC level with 4 samples each day.

5.2.5. Analyze two calibration curves together with the QC samples on each day.

5.2.6. Calculate intra-day accuracy as the % of the nominal concentration (six samples per concentration level, please see 5.2.2). Calculate precision as the coefficient of variance (CV%).

5.2.7. Consider intra-day accuracy acceptable if it falls into the acceptance limits 85% to 115% of the nominal concentration. Consider intra-day precision acceptable if it is equal to or better than a CV (coefficient of variance) of 15%.

5.2.8. Calculate inter-day accuracy and precision as the mean for each QC concentration level analyzed over the 20 validation days.

5.2.9. Consider mean inter-day accuracy acceptable if it falls into the acceptance limits 85% to 115% of the nominal concentration. Consider inter-day precision acceptable if it is equal to or better than a CV (coefficient of variance) of 15%.

5.3. Exclusion of matrix interferences.

5.3.1. For the exclusion of interferences that may be caused by matrix signals, analyze blank dried blood spots (8 different individuals, preferably 4 males and 4 females).

5.3.2. Visually inspect ion chromatograms. If peaks within the retention time window of tacrolimus are detected, integrate and compare their areas under the curve with those of tacrolimus peaks in blank samples spiked with tacrolimus at the LLOQ. The area of peaks in the blank samples are not supposed to exceed 15% of those of tacrolimus at the LLOQ.

5.4. Ion suppression/ ion enhancement.

5.4.1. Use a post-column infusion protocol as described[45] to assess potential interference of ion suppression/ ion enhancement caused by co-eluting matrix components.

5.4.2. Infuse tacrolimus at a concentration of 10 µg/mL dissolved in 0.1% formic acid: methanol (30:70, v/v) post-column at a rate of 10 µL/min.

5.4.2.1. Connect a syringe pump via T-piece between the analytical column and the electrospray source of the mass spectrometer.

5.4.2.2. Monitor the MS/MS signal intensity of the MRM transitions for tacrolimus and its internal standard (m/z = 826.6 → 616.2 and m/z = 829.6 → 619.2) after injection of extracted blank samples (n = 8 samples from different individuals).

Note: In the absence of ion suppression/ ion enhancement the continuous signal caused by infusion of the analytes should not be affected by injection of the blank matrix, while ion suppression causes a dip of the signal and ion enhancement a peak.

5.5. Carry-over.

5.5.1. Assess potential carry-over by analyzing extracted blank samples after the highest calibrators (50 ng/mL, n = 6).

5.5.2. Visually inspect ion chromatograms. If peaks within the retention time window of tacrolimus are detected, integrate and compare their areas under the curve with those of tacrolimus peaks in blank samples spiked with tacrolimus at the LLOQ. The area of peaks in the blank samples are not supposed to exceed 15% of those of tacrolimus at the LLOQ.

5.6. Extraction recoveries.

5.6.1. Determine recoveries by comparing the signals of the analytes after extraction of QC samples at all four concentration levels (n = 6 per concentration) with those of blank dried blood spots spiked with the corresponding amounts of tacrolimus after the extraction.

5.6.2. Prepare four sets of QCs (concentration levels: 2, 4, 20, 40 ng/mL).

5.6.3. Prepare another 4 sets of corresponding “recovery test samples” by spotting 50 µL of blank EDTA whole blood onto the filter paper cards and drying for 2 h.

5.6.4. Hereafter, for both the QC and blank “recovery test samples”, cut out the entire blood spot on the filter card with scissors and place the resulting discs into a polypropylene tube with conical bottom and snap-on lid.

5.6.5. Extract all samples.

5.6.6. Transfer the supernatants (400 µL) into glass HPLC vials.

5.6.7. Add tacrolimus stock solution to the blank “extracted recovery test samples” to reach concentrations of 2, 4, 20 and 40 ng/mL (4 µL of 200, 400, 2000, 4000 ng/mL tacrolimus stock solutions to 400 µL of supernatant).

5.6.8. After LC-MS/MS analysis, compare the signals in both QC samples and “recovery test samples” of the corresponding concentration (recovery%= signal samples spiked before extraction/ signal samples spiked after extraction x 100).

5.7. Dilution integrity.

5.7.1. Establish dilution integrity using samples spiked with the analytes at 500, 250 and 100 ng/mL.

5.7.2. After extraction, dilute samples using protein precipitation solution (1:10, n = 3 per concentration level).

5.7.3. Calculate deviations from the nominal concentrations. Consider results that fall within 85-115% of nominal acceptable.

5.8. Stabilities.

5.8.1. Investigate stabilities using the QC samples at all four concentration levels (n = 4 per concentration) analyzed at different time-points and under the different storage conditions.

5.8.2. Compare results after storage with the nominal values. Consider results that fall within 85-115% of nominal acceptable.

5.8.3. Establish sample stability for 1 week at ambient temperature, 1 week at 4 °C, 1 month at -20 °C and 1 month at -80 °C.

5.8.4. Test freeze-thaw stability over three cycles (-20 °C). Test extracted sample and autosampler stability by placing samples into the thermostatted autosampler adjusted to 4 °C. Inject samples after 72 h.

Representative Results:
Representative ion chromatograms of a blank sample, a sample spiked at the lower limit of quantification and a patient sample are shown in Figure 3.

Calibration Curves
The lower limit of detection was 0.5 ng/mL and the lower limit of quantification was 1.0 ng/mL. Fifty ng/mL was chosen as the highest calibrator as higher concentrations are unlikely to be reached in the clinic under normal circumstances.

Calibration curves were freshly prepared on each validation day in human EDTA whole blood, dried on filter cards and extracted with methanol / 0.2 M $ZnSO_4$ (70:30 v/v) + internal standard (final concentration of internal standard: 2.5 ng/mL). For the day 1 validation (n = 6 for calibrators and n = 6 for QC level) and for days 2 - 20 (n = 2 for calibrators and n = 4 for each QC level) were analyzed with concentrations 1, 2.5, 5, 10, 25, 50 ng/mL for calibrators. A typical calibration curve is shown in Figure 4. Mean accuracies of 85% to 115% of nominal, within the working range for 2/3 of the calibrators (with a minimum of 6 non-zero calibrators) were considered acceptable. The mean coefficient of correlation was (r) = 0.999 (n = 40 calibration curves).

Accuracies and Precisions
The results are shown in detail in Table 3.

Extraction Recovery
Average extraction recoveries were 98.2% (2 ng/mL), 92.2 (4 ng/mL), 95.5 (20 ng/mL), 96.2 (40 ng/mL).

Matrix Interferences, Ion Suppression/ Ion Enhancement Testing Using Continuous Post-Column Infusion and Carry-over
Analysis of the blank samples from eight different individuals (n = 4 female and n = 4 male) showed signals less than 15% of the LLOQ (1 ng/mL) at the retention time corresponding to the tacrolimus peak indicating that the detected tacrolimus peak can be considered specific. A representative example is shown in Figure 3. Potential interferences by ion suppression/ ion enhancement were tested using blank dried blood samples from eight different healthy individuals. A representative experiment is shown in Figure 5. No indications of significant ion suppression/ ion enhancement

were observed. No relevant carry-over resulting in peaks exceeding 15% of the signal at LLOQ were detected.

Dilution Integrity

Dilution integrity was investigated by analyzing samples prepared at concentrations above the highest calibrator (100, 250 and 500 ng/mL) and diluted 1:10 in protein precipitation solution after extraction to reach the target concentrations of: 10, 25, and 50 ng/mL. Mean accuracies had to fall within the acceptance criteria of 85% to 115% of the nominal concentrations. All dilutions tested met acceptance criteria (Table 4).

Stabilities

Stability of tacrolimus in dried blood spots was investigated by analyzing QC samples at all four levels (n= 4/ concentration level), which were stored under varying conditions. Mean accuracies had to fall within the acceptance criteria of 85% to 115% of the nominal concentrations. Results are shown in detail in Table 5. No losses after 1 week of storage at room temperature, after 1 week of storage at 4 °C, after 1 month of storage at -20 °C, after 1 month of storage at -80 °C, after 3 freeze and thaw cycles, and after 72 h of extracted samples in auto sampler at 4 °C were apparent.

Table 1. *Gradient Program for the Extraction and Analytical HPLC Pumps.*

Table 2. *Turbo Electrospray Interface and Mass Spectrometer Parameters.* The nomenclature corresponds to that used in the mass spectrometry software (for manufacturer details, please see Table of Materials and Equipment).

Table 3. *Results of Quality Control Samples over 20 Days.* Data is presented as % of nominal. Samples listed as “failed” are samples that were lost to laboratory/ instrument errors. In most cases, no peaks were detected at all or the internal standard peak was missing.

Table 4. *Results of Dilution Integrity Testing.* Data is presented as % of nominal.

Table 5. *Results of Stability Testing.* 5A: Stability of tacrolimus on dried blood spots at room temperature over 7 days, 5B: Stability of tacrolimus on dried blood spots in the refrigerator (+4 °C) over 7 days, 5C: Stability of tacrolimus on dried blood spots at -20 °C over 1 month, 5D: Stability of tacrolimus on dried blood spots at -80 °C over 1 month, 5E: Stability of tacrolimus on dried blood spots over three freeze-thaw cycles (-20°C), 5F: Extracted sample/ autosampler stability at +4 °C over 72 hours. Data is presented as% of nominal concentration. Samples listed as “failed” are samples that were lost to laboratory/ instrument errors. In most cases no peaks were detected at all or the internal standard peak was missing.

Figure 1. *Structure of Tacrolimus.* Atom numbering follows the International Union of Pure and Applied Chemistry (IUPAC) nomenclature.

Figure 2. *Connection of the Switching Valve.*

Figure 3. *Representative Ion Chromatograms.* 3A: Representative ion chromatogram of a blank blood samples spotted onto filter paper (for manufacturer details, please see Table of Materials and Equipment) and dried. The arrow marks the retention time of the tacrolimus peak, 3B Representative ion chromatogram of a blank blood samples spiked at the lower limit of quantification (1 ng/mL) spotted onto the filter paper and dried, and 3C: Representative ion chromatogram of a sample collected by a transplant patient on filter paper. This is a trough sample and the measured tacrolimus concentration was 2.1 ng/mL. This sample was collected by the patient at home and is from a clinical trial that was approved by the University of Cincinnati Institutional Review Board (Cincinnati, OH). All patients gave their appropriate written consent. Ion chromatograms are original print-outs as generated by the mass spectrometry software (for manufacturer details, please see Table of Materials and Equipment). Blue and red lines in ion chromatograms represent the internal standard D_2,^{13}C-tacrolimus and tacrolimus, respectively. The peak eluting in front of the main tacrolimus and internal standard peaks are the rotamers.

Figure 4. *Representative Calibration Curve.* An original print-out as generated by the mass spectrometry software is shown.

Figure 5. *Representative Ion Chromatogram Measured During Post-Column Infusion of Tacrolimus and Injection of an Extracted Blank Blood Sample to Assess a Potential Matrix Effect (Ion Suppression/ Ion Enhancement).* The arrow marks the retention time of the tacrolimus peak. Matrix effect testing was based on the procedure described in[46]. No relevant matrix effect was detected.

Discussion:

Although, as aforementioned, the concept of therapeutic drug and adherence monitoring of tacrolimus based on dried blood spots is attractive, there are analytical challenges that go beyond those typically associated with the LC-MS/MS analysis of tacrolimus in venous EDTA whole blood samples. These include, but are not limited to, the fact that the matrix is capillary whole blood soaked into the cotton linters material of the filter card material used here and the low blood volume (20 µL). Nevertheless, high-throughput analysis in a central laboratory requires a fast and reliable extraction method that results in samples that lack matrix interferences and matrix effects in combination with a robust, specific and highly sensitive LC-MS/MS assay. Reliability of the assay is critical as there is usually not enough material on the already punched filter card left for re-analysis in case the extraction/ LC-MS/MS analysis fails.

The filter cards used in the present study (for manufacturer details, please see Table of Materials and Equipment) were chosen as these are an FDA approved class II device, are in compliance with NCCLS guidance LA4-A5[44] and are CE-marked in Europe. If completely filled, one circle on the Whatman 903 filter card holds ≈50 µL blood[46]. However, the size of the blood drops collected by individual patients vary and training in the proper sampling technique is essential[46].

The first important step of extracting a punched-out dried blood spot sample is homogenization. Based on our experience, the use of a bullet blender is more efficient and better reproducible than other methods used to enhance extraction efficiency such as sonication. The use of the bullet blender was essential to consistently achieve extraction recoveries above 90%. For reliability of the extraction procedure, it was also important to ensure that all centrifuges were temperature controlled (4 °C) and that the vortexing step was not shorter than 10 min, which resulted in more variable and lower extraction recoveries. In addition, it is important that the methanol/ $ZnSO_4$ ratio is not altered as tacrolimus recovery is very sensitive to the correct composition of the protein precipitation solution.

The next challenge is to obtain a clean extract ideally devoid of materials that may cause matrix interferences and effects. Thus, a simple one-step protein precipitation step as often used for the extraction of tacrolimus from EDTA blood samples was not considered a viable option. After protein precipitation using $ZnSO_4$ (including addition of an isotope-labeled internal standard), vortexing and centrifugation, the supernatants were injected into a 2D HPLC system and onto an online extraction column. Online column extraction using high flows of 5 mL/min on conventional pre-column cartridges using a simple 6-port switching valve for the analysis of tacrolimus have been described before[47]. The mobile phase was chosen so that tacrolimus and its internal standard concentrated in the front of the extraction column and did not migrate over the column during the cleanup step. The online extraction used in the present protocol had several advantages including injection of relatively large sample volumes without negatively affecting HPLC analysis. The back-flush after enriching the analytes on top of the extraction column (“peak focusing”) resulted in sharper peaks allowing for more reliable integration by the software algorithm especially for samples with low tacrolimus concentrations.[48] The online cleanup step did not only remove potentially interfering matrix compounds, but also de-salted the sample. One important yet rarely discussed problem for high-volume, high throughput LC-MS/MS assays is the gradual loss of sensitivity of the LC-MS/MS system due to increasing contamination of the electrospray source during analysis of large batches. No significant matrix effects (ion suppression/ ion enhancement) were observed. The negative effect of potential matrix effects were reduced/ avoided by combination of the following: effective protein precipitation using methanol/ $ZnSO_4$, centrifugation after protein precipitation at 16,000 x g, high-flow online extraction, clear chromatographic separation of tacrolimus from potential interferences early eluting from the analytical column and the use of isotope-labeled tacrolimus as internal standard instead of structurally related internal standards such as ascomycin.

The lower limit of quantification was 1 ng/mL, and thus lower than that of most immunoassays that are currently frequently used for therapeutic drug monitoring of tacrolimus in EDTA blood samples. This lower limit of quantification is sufficient even for so-called low calcineurin inhibitor long-term immunosuppressive maintenance protocols.

In comparison with previously described LC-MS/MS assays to quantify tacrolimus in dried bloods spots[29-34,36,39,41,42], the present assay matches or exceeds their performance in terms of lower limit of quantification, extraction recovery, accuracy and precision while avoiding potentially risky concepts such as one-step protein precipitation procedures and ultra-short chromatography times, which usually give acceptable results during the validation based on blood samples from healthy individuals. However, transplant patients are a highly complex group of patients who have diseases that affect the composition of blood and who take multiple medications. This makes it virtually impossible to exclude all potential interferences that may be present in individual patients during the validation and the only viable strategy is to set up the assay in a way that it minimizes the risk of such potential interferences. Dried blood spots have challenges such as the effect of the hematocrit on blood viscosity and thus the diffusion properties of the blood applied on filter paper[49]. This was not tested here again as such effects have already been described not to affect tacrolimus analysis in dried blood spots at hematocrits and tacrolimus concentrations within clinically reasonable limits[29,32]. Also stability of tacrolimus in dried blood spots at elevated temperatures has already been studied by others and tacrolimus in dried blood spots was found to be stable for 5 days at 37 °C and even 60 °C[32], which is important for shipment of dried blood spots under not temperature-controlled conditions especially during summer.

This assay based on a combination of bullet blender homogenization, high-flow online column cleanup and LC-MS/MS analysis may provide a platform strategy for the development of bioanalytical assays for the quantification of other immunosuppressants, alone or simultaneous, as well as of other drugs in dried blood spot samples.

Acknowledgments:
This work was supported by the United States Federal Drug Administration (FDA) contract HHSF223201310224C and the United States National Institutes of Health/FDA grant 1U01FD004573-01.

Disclosures:
The authors have nothing to disclose.

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Figure

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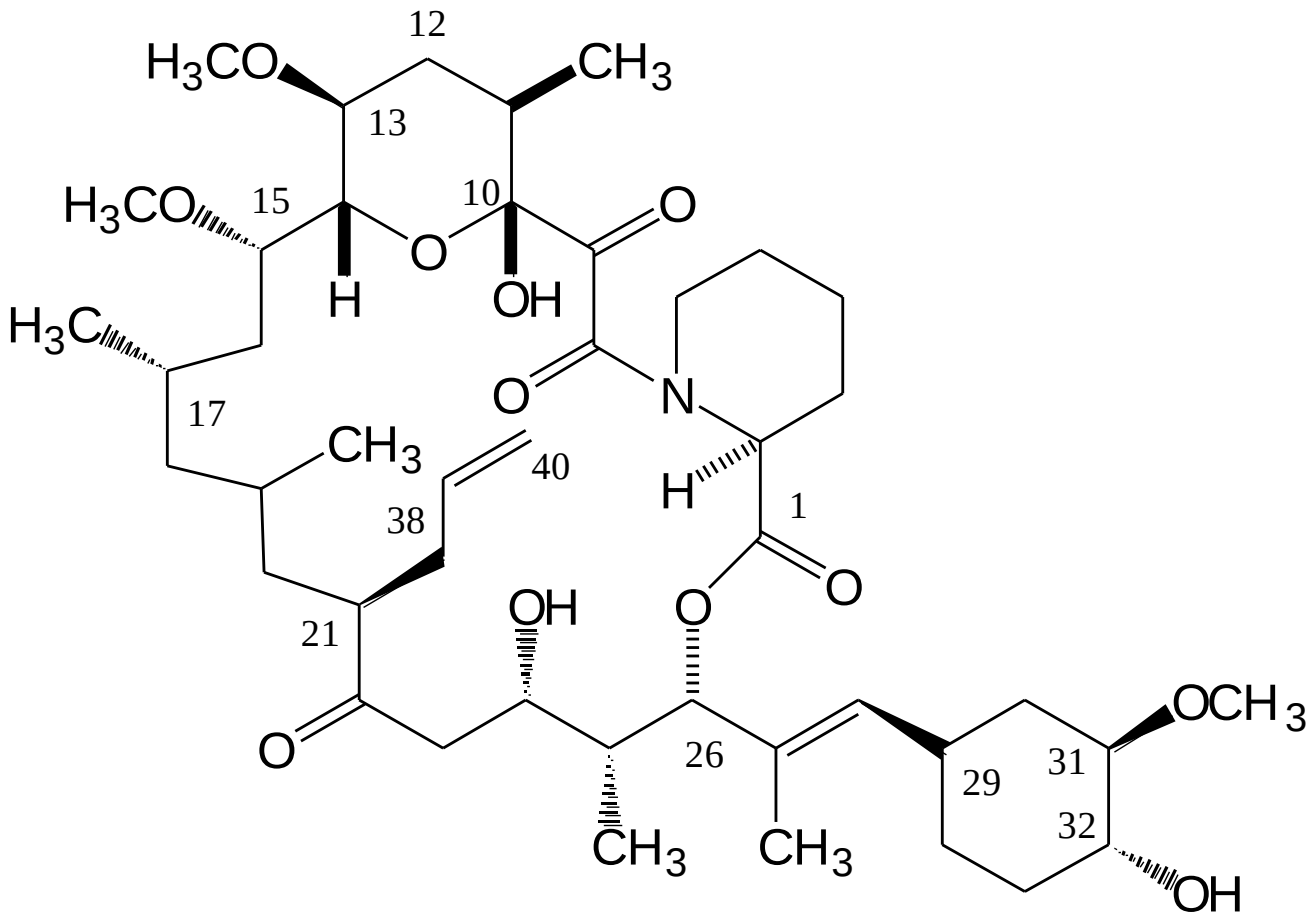

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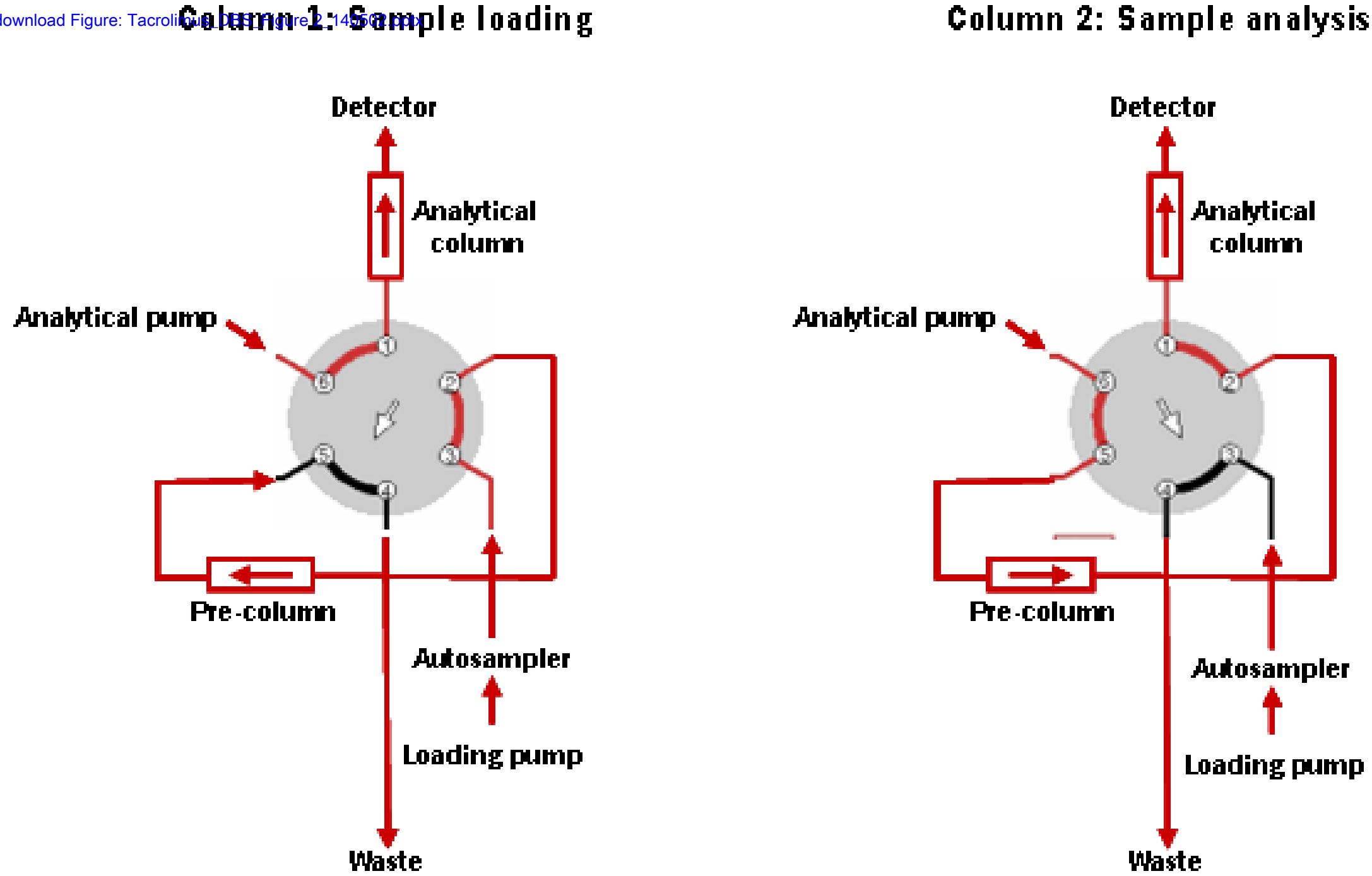

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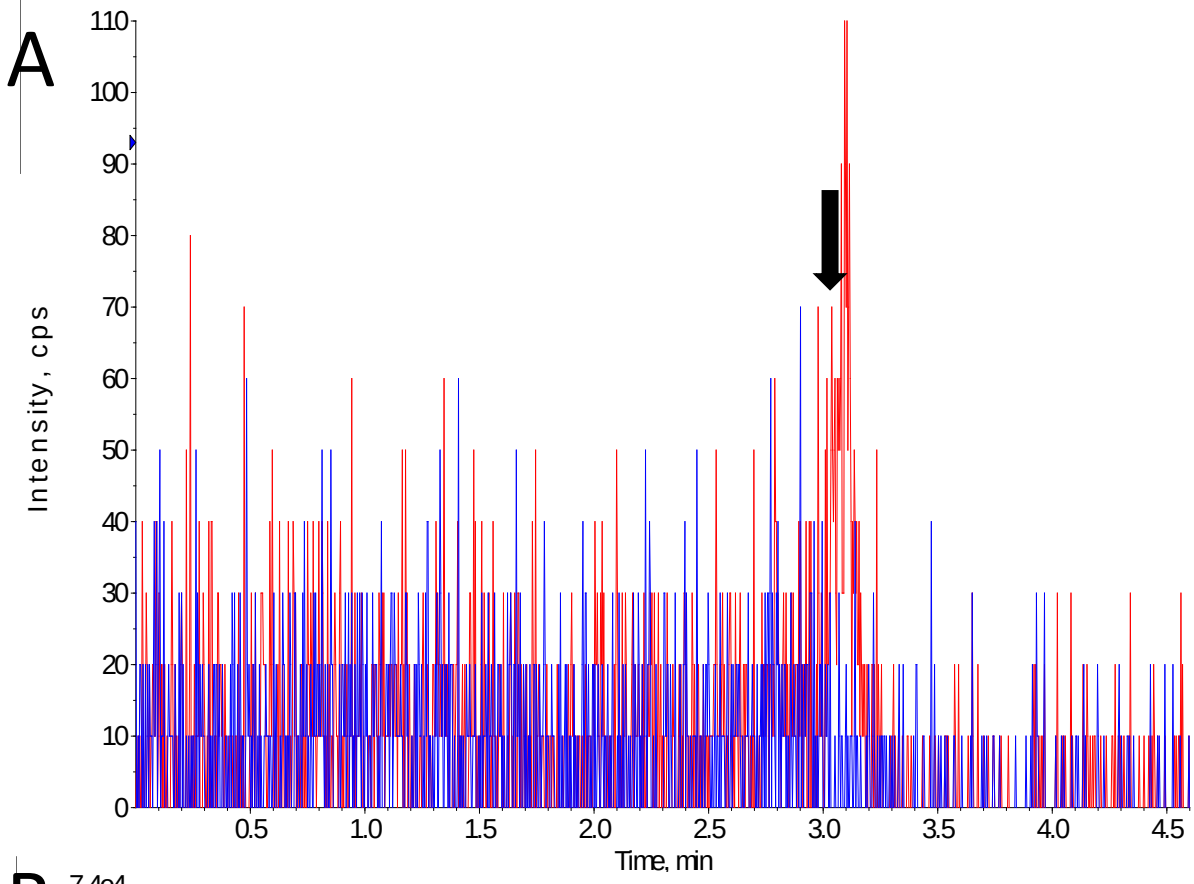

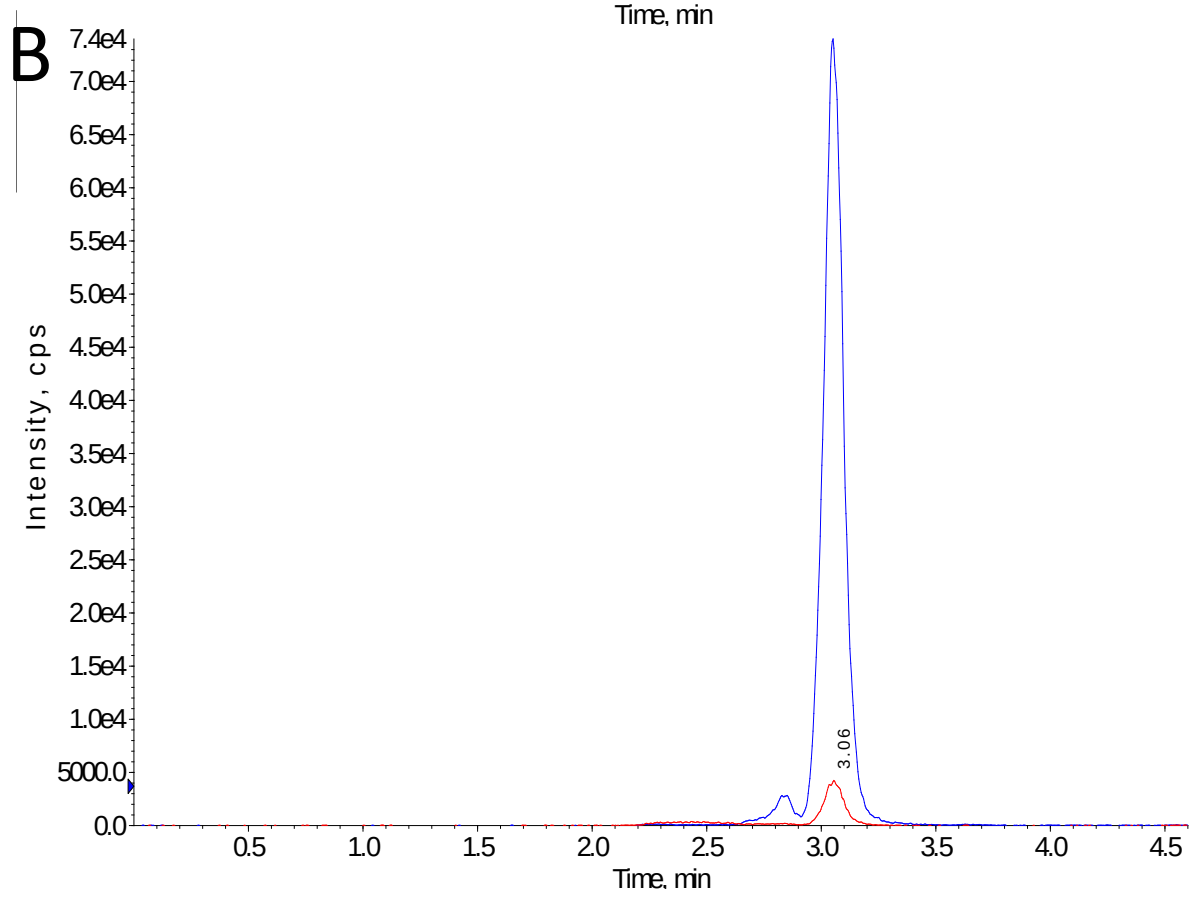

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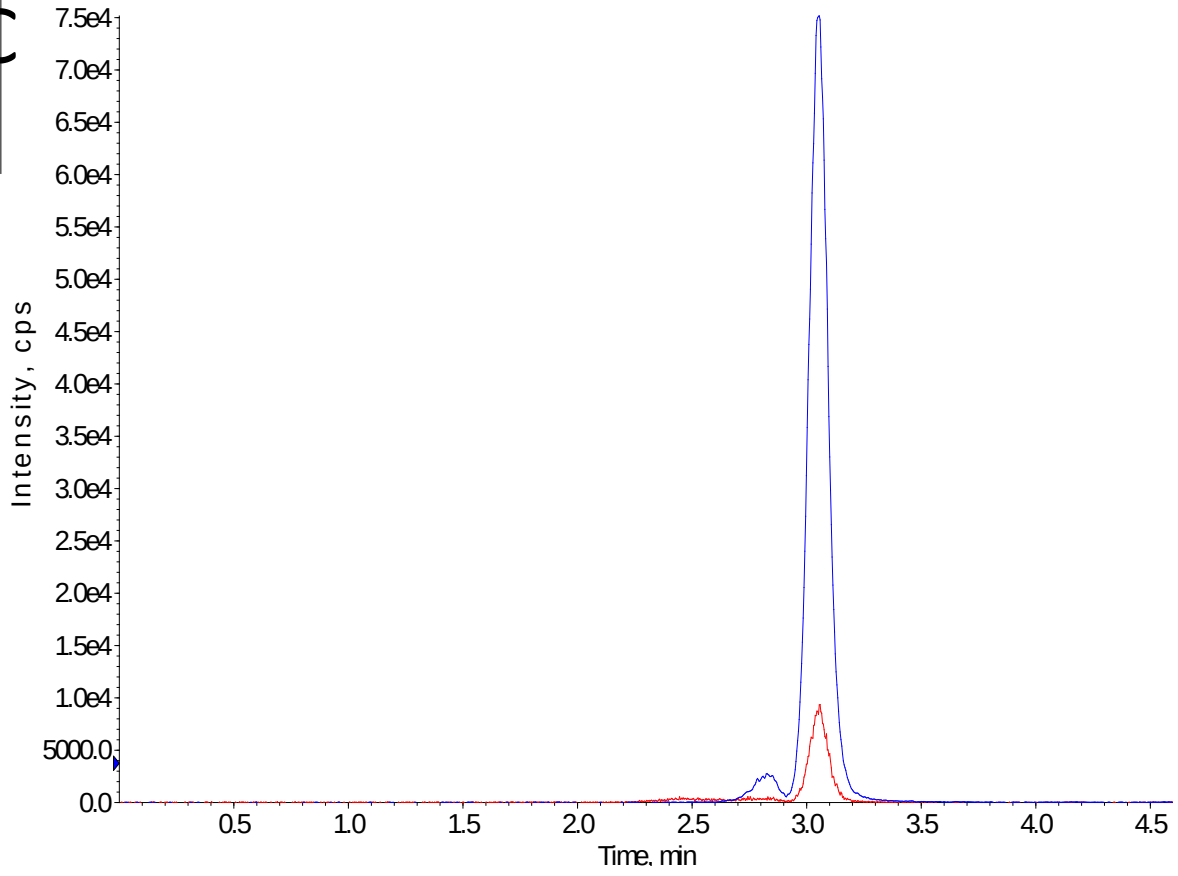
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2.5e4
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1.5e4
1.0e4
5000.0
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Intensity, cps
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time, min

Figure

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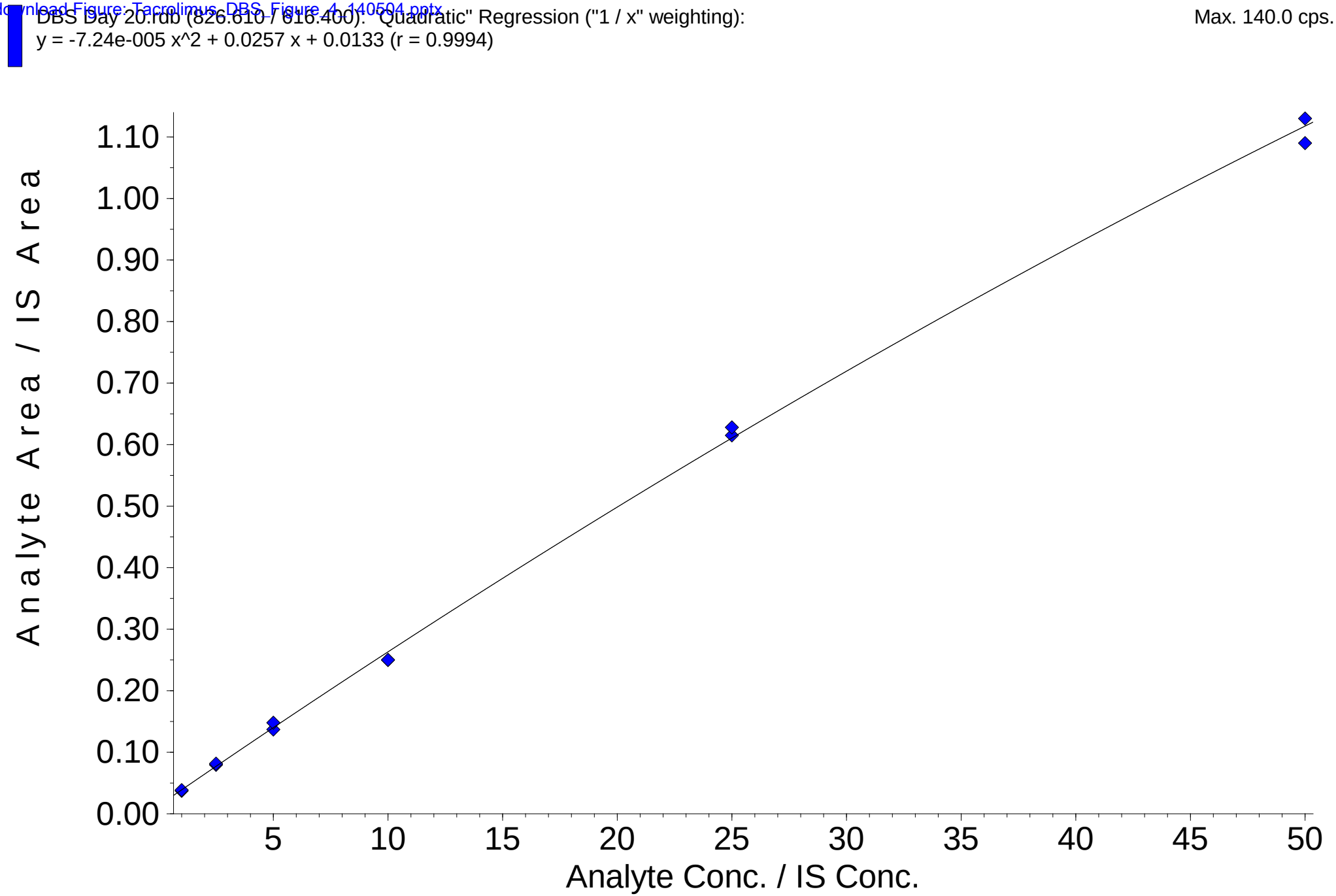

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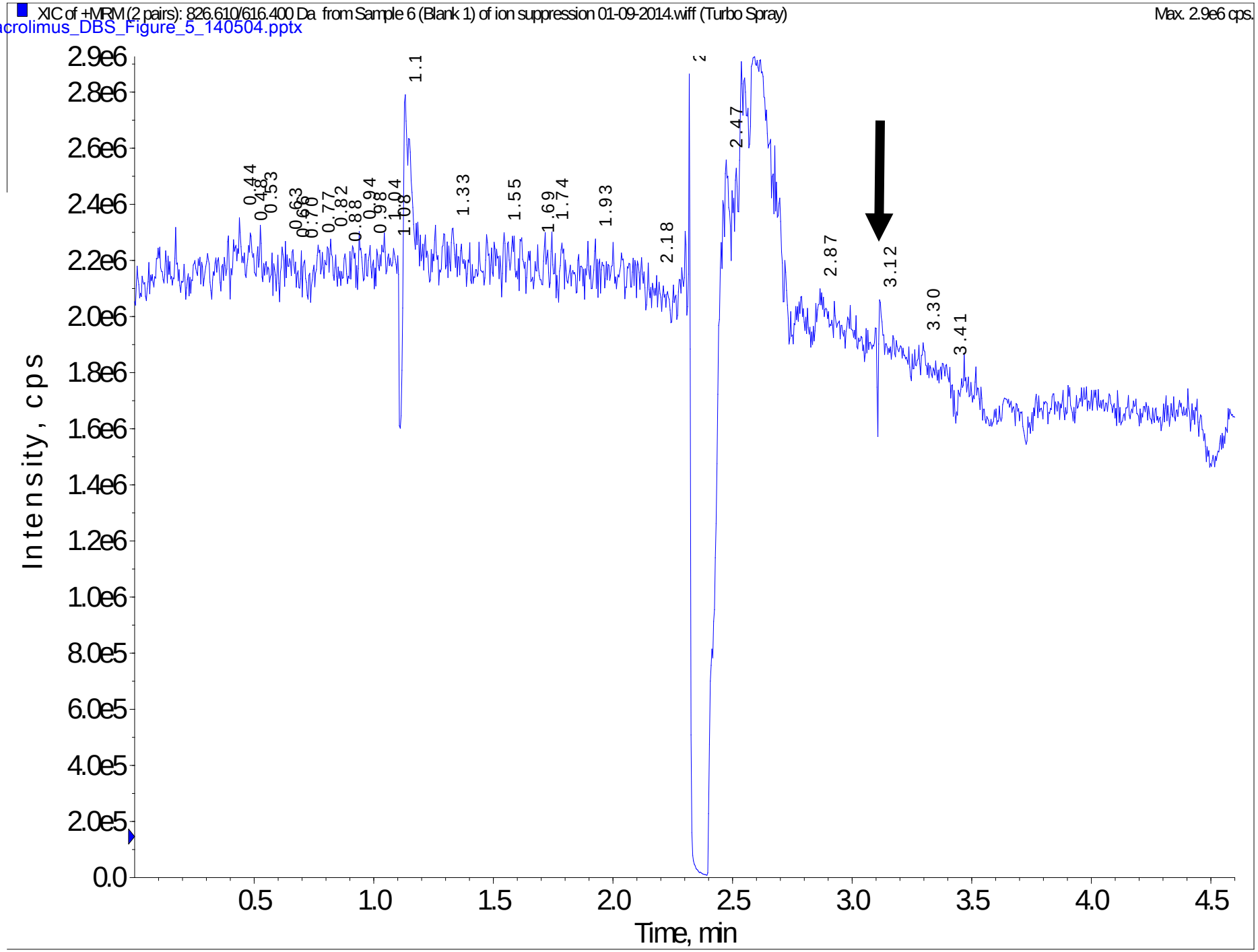

Table

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Extraction Pump				Analytical (Elution) Pump			
Time [min]	Water + 0.1% formic acid	Acetonitrile	Flow rate [µL/min]	Time [min]	Water + 0.1% formic acid	Acetonitrile	Flow rate [µL/min]
0	70	30	5000	0	13	87	1000
1	70	30	5000	2	2	98	1200
1.1	2	98	100	3.5	2	98	1200
3	2	98	100	3.6	13	87	1000
3.1	20	80	2000	4.6	13	87	1000
4	70	30	5000				
4.6	70	30	5000				

Table

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Parameter	Setting
Collision gas (CAD)	10
Curtain gas (CUR) (psi)	30
Ion Source gas 1 (GS1) (psi)	50
Ion source gas 2 (GS2) (psi)	30
Nebulizer current (NC) (V)	1
Temperature (TEM) (°C)	600
IonSpray Voltage (IS) (V)	5500
Interface heater (ihe)	On
Declustering potential (DP) (V)	136
Entrance potential (EP) (V)	10
Collision energy (CE) (V)	47
Collision cell exit potential (CXP) (V)	16

Table

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Validation Day	QC Level [% of nominal concentration]			
	2	4	20	40
Day 1	93.0	86.3	88.9	93.4
	101	90.4	95.3	100
	85.6	95.9	99.0	97.5
	88.6	93.6	105	103
	85.0	97.4	97.2	109
	89.1	96.7	100	101
Intra-day Accuracy [%]	90.4	93.4	97.6	100.7
Intra-Day Imprecision [CV%]	6.6	4.6	5.5	5.2
Day 2	92.8	86.0	103	95.4
	91.1	88.8	94.7	87.0
	88.6	90.9	92.8	94.2
	97.2	93.7	94.2	115
Intra-day Accuracy [%]	92.4	89.9	96.2	97.9
Intra-Day Imprecision [CV%]	3.9	3.6	4.8	12.2
Day 3	97.6	101	98.4	112
	104	85.6	102	110
	99.2	88.4	99.4	105
	96.3	87.2	108	117
Intra-day Accuracy [%]	99.3	90.6	102.0	111.0
Intra-Day Imprecision [CV%]	3.4	7.8	4.2	4.5
Day 4	95.2	88.6	112	94.5
	105	87.3	93.2	116
	99.8	96.2	103	103
	100	104	97.2	99.4
Intra-day Accuracy [%]	100.0	94.0	101.4	103.2
Intra-Day Imprecision [CV%]	4.0	8.2	8.1	8.9
Day 5	106	90.4	101	106
	108	89.0	106	100
	102	101	96.6	128
	105	88.8	105	107
Intra-day Accuracy [%]	105.3	92.3	102.2	110.3
Intra-Day Imprecision [CV%]	2.4	6.3	4.2	11.1
Day 6	90.9	93.7	119	106
	98.8	88.1	96.4	110
	94.6	96.3	99.1	108
	108	100	102	102
Intra-day Accuracy [%]	98.1	94.5	104.1	106.5
Intra-Day Imprecision [CV%]	7.5	5.3	9.8	3.2

	85.1	87.5	99.5	95.4
Day 7	86.4	85.4	94.7	101
	94.5	87.3	98.9	94.6
	85.5	97.0	101	99.6
Intra-day Accuracy [%]	87.9	89.3	98.5	97.7
Intra-Day Imprecision [CV%]	5.1	5.8	2.7	3.2
	86.1	92.5	91.9	102
Day 8	87.5	91.5	95.2	88.5
	115	85.6	92.1	102
	85.8	85.9	95.4	108
Intra-day Accuracy [%]	93.6	88.9	93.7	100.1
Intra-Day Imprecision [CV%]	15.3	4.1	2.0	8.2
	88.9	91.4	96.9	100
Day 9	90.0	89.8	95.0	100
	69.7	85.9	95.8	109
	91.9	87.0	105	101
Intra-day Accuracy [%]	85.1	88.5	98.2	102.5
Intra-Day Imprecision [CV%]	12.2	2.9	4.7	4.3
	90.9	91.3	96.2	100
Day 10	97.7	89.5	94.4	100
	99.9	109	98.7	96.8
	99.1	90.0	95.7	96.1
Intra-day Accuracy [%]	96.9	95.0	96.3	98.2
Intra-Day Imprecision [CV%]	4.2	9.9	1.9	2.1
	92.7	91.9	88.2	104
Day 11	96.6	91.2	97.0	110
	109.0	92.8	97.6	102
	98.3	107	93.7	111
Intra-day Accuracy [%]	99.2	95.7	94.1	106.8
Intra-Day Imprecision [CV%]	7.0	7.9	4.6	4.1
	87.7	85.5	105	95.3
Day 12	112	88.1	101	96.1
	102	89.1	89.7	97.5
	106	92.5	102	104
Intra-day Accuracy [%]	101.9	88.8	99.4	98.2
Intra-Day Imprecision [CV%]	10.1	3.3	6.7	4.0
	Failed	85.7	93.3	102
Day 13	101	105	88.0	93.9
	112	98.0	91.4	102
	104	113	104	101
Intra-day Accuracy [%]	105.7	100.4	94.2	99.7
Intra-Day Imprecision [CV%]	5.4	11.5	7.3	3.9

	91.5	89.1	97.4	93.1
Day 14	90.4	87.1	93.9	99.8
	89.7	97.0	94.8	106
	97.4	86.8	89.9	Failed
Intra-day Accuracy [%]	92.3	90.0	94.0	99.6
Intra-Day Imprecision [CV%]	3.8	5.3	3.3	6.5
	92.8	92.8	92.5	95.4
Day 15	97.5	96.3	96.2	95.5
	95.5	108	97.3	99.3
	110	109	115	113
Intra-day Accuracy [%]	99.0	101.5	100.3	100.8
Intra-Day Imprecision [CV%]	7.7	8.1	10.0	8.3
	93.7	97.8	90.7	112
Day 16	90.3	87.1	Failed	101
	97.9	88.3	95.5	107
	91.4	85.7	89.3	96.7
Intra-day Accuracy [%]	93.3	89.7	91.8	104.2
Intra-Day Imprecision [CV%]	3.6	6.1	3.5	6.4
	88.0	86.0	93.7	103
Day 17	89.8	90.8	94.8	93.2
	85.9	91.1	99.7	94.8
	86.7	88.1	95.6	91.7
Intra-day Accuracy [%]	87.6	89.0	96.0	95.7
Intra-Day Imprecision [CV%]	1.9	2.7	2.7	5.3
	89.6	85.8	91.0	98.3
Day 18	89.6	86.2	88.3	93.6
	Failed	86.7	96.8	104
	88.1	85.8	95.2	111
Intra-day Accuracy [%]	89.1	86.1	92.8	101.7
Intra-Day Imprecision [CV%]	1.0	0.5	4.2	7.4
	98.0	89.7	94.2	102
Day 19	88.3	86.0	97.6	102
	91.6	88.1	95.8	97.5
	90.7	90.1	92.8	88.3
Intra-day Accuracy [%]	92.2	88.5	95.1	97.5
Intra-Day Imprecision [CV%]	4.5	2.1	2.2	6.6
	93.0	87.0	99.4	101
Day 20	97.3	87.6	95.5	91.9
	89.0	88.4	91.2	93.5
	104	90.7	97.7	115
Intra-day Accuracy [%]	95.8	88.4	96.0	100.4
Intra-Day Imprecision [CV%]	6.7	1.8	3.7	10.5

Inter-Day Accuracy and Imprecision

Inter-day Accuracy	**95.2**	**91.7**	**97.2**	**101.6**
Inter Day Precision	**6.1**	**4.5**	**3.6**	**4.2**

Table

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Dilution	1:10
Nominal target concentration after dilution	50 ng/mL
	98.6
	94.5
	91.4
Accuracy [%]	94.8
Imprecision [CV%]	3.6

Dilution	1:10
Nominal target concentration after dilution	10 ng/mL
	103
	99.5
	101
Accuracy [%]	101.2
Imprecision [CV%]	1.8

Dilution	1:10
Nominal target concentration after dilution	25 ng/mL
	91.7
	98.2
	103
Accuracy [%]	97.6
Imprecision [CV%]	5.7

Table

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Stability at room temperature, Day 1

QC level [ng/mL]	2	4	20	40
	91.9	85.8	86.0	102
	86.7	85.7	88.5	102
	86.0	85.9	90.1	103
	89.2	98.0	90.8	112
% of nominal concentration	88.5	88.9	88.9	104.8
Imprecision [%CV]	2.7	6.1	2.1	4.9

Stability at room temperature, Day 3

QC level [ng/mL]	2	4	20	40
	88.6	105	101	113
	94.1	100	98.5	103
	100	101	106	109
	99.5	102	102	108
% of nominal concentration	95.6	102.0	101.9	108.3
Imprecision [%CV]	5.3	2.2	3.1	4.1

Stability at room temperature, Day 7

QC level [ng/mL]	2	4	20	40
	105	103	91.7	109
	101	107	100	110
	102	108	107	105
	93.8	105	109	111
% of nominal concentration	100.5	105.8	101.9	108.8
Imprecision [%CV]	4.7	2.2	7.8	2.6

Stability at +4°C, Day 1

QC level [ng/mL]	2	4	20	40
	101	89.9	95.8	100
	88.9	91.0	94.1	99.0
	96.2	100	102	96.7
	89.5	87.8	95.4	88.6
% of nominal concentration	93.9	92.2	96.8	96.1
Imprecision [%CV]	5.8	5.4	3.5	5.2

Stability at +4°C, Day 3

QC level [ng/mL]	2	4	20	40
	87.3	85.2	105	95.3
	Failed	87.8	101	96
	101	88.8	89.6	97.5
	106	92.2	102	104
% of nominal concentration	98.1	88.5	99.4	98.2
Imprecision [%CV]	9.7	2.9	6.8	4.0

Stability at +4°C, Day 7

QC level [ng/mL]	2	4	20	40
	94.0	98.5	96.1	110
	92.9	96.4	109	109
	91.7	96.3	97.9	115
	94.7	96.9	99.8	113
% of nominal concentration	93.3	97.0	100.7	111.8
Imprecision [%CV]	1.3	1.0	5.7	2.8

Stability at -20°C, Day 3

QC level [ng/mL]	2	4	20	40
	87.3	98.7	111	111
	93.5	89.6	108	105
	89.5	91.5	107	112
	88.2	99.1	108	92.4
% of nominal concentration	89.6	94.7	108.5	105.1
Imprecision [%CV]	2.7	4.9	1.7	9.0

Stability at -20°C, Day 7

QC level [ng/mL]	2	4	20	40
	96.5	98.7	102	109
	94.8	94.6	114	106
	95.5	102	98.1	108
	107	99.5	115	105
% of nominal concentration	98.5	98.7	107.3	107
Imprecision [%CV]	5.7	3.1	8.5	1.8

Stability at -20°C, Day 30

QC level [ng/mL]	2	4	20	40
	82.3	83.1	90.4	93.2
	87.9	85.8	85.3	97.9
	85.7	88.6	98.3	98.0
	92.0	95.6	110	103
% of nominal concentration	87.0	88.3	96.0	98.0
Imprecision [%CV]	4.1	5.4	10.8	4.0

Stability at -80°C, Day 3

QC level [ng/mL]	2	4	20	40
	87.5	96.5	96.7	Failed
	Failed	97.6	98.7	110
	88.8	96.4	106	109
	87.3	101	96.5	109
% of nominal concentration	87.9	97.9	99.5	109.3
Imprecision [%CV]	0.8	2.2	4.5	0.6

Stability at -80°C, Day 7

QC level [ng/mL]	2	4	20	40
	Failed	98.0	105	99.8
	97.1	106	104	105
	99.7	102	99.3	102
	101	99.9	109	106
% of nominal concentration	99.3	101.5	104.3	103.2
Imprecision [%CV]	2.0	3.4	4.0	2.8

Stability at -80°C, Day 30

QC level [ng/mL]	2	4	20	40
	88.2	85.3	89.6	96.7
	96.2	92.6	85.8	94.1
	83.9	93.7	91.0	105
	95.6	94.0	98.9	102
% of nominal concentration	91.0	91.4	91.3	99.5
Imprecision [%CV]	6.0	4.1	5.5	4.9

Freeze thaw stability, -20°C, 1 cycle

QC level [ng/mL]	2	4	20	40
	92.5	86.2	90.2	94.3
	89.8	90.5	85.4	104
	94.7	88.6	93.4	104
	101	89.2	93.7	96.3
% of nominal concentration	94.5	88.6	90.7	99.7
Imprecision [%CV]	4.8	1.8	3.9	5.1

Freeze thaw stability, -20°C, 2 cycles

QC level [ng/mL]	2	4	20	40
	99.1	97.6	Failed	85.3
	93.1	88.1	93.4	92.0
	94.9	91.5	85.8	93.9
	Failed	90.5	86.8	85.4
% of nominal concentration	95.7	91.9	88.7	89.2
Imprecision [%CV]	3.1	4.0	4.1	4.5

Freeze thaw stability, -20°C, 3 cycles

QC level [ng/mL]	2	4	20	40
	95.7	Failed	86.0	93.3
	95.5	87.4	85.0	91.3
	90.5	89.1	86.0	86.0
	96.9	85.7	90.2	Failed
% of nominal concentration	94.7	87.4	86.8	90.2
Imprecision [%CV]	2.8	1.7	2.3	3.8

Extracted sample stability at +4°C, 24 h

QC level [ng/mL]	2	4	20	40
	122	112	91.0	104
	93.5	106	91.8	96.1
	111	94.8	98.2	93.5
	98.4	97.6	91.3	89.8
% of nominal concentration	106.2	102.6	93.1	95.9
Imprecision [%CV]	12.8	7.9	3.4	6.0

Extracted sample stability at +4°C, 48 h

QC level [ng/mL]	2	4	20	40
	106	108	93.7	110
	105	98.1	94.6	98.9
	103	98.4	95.0	92.6
	108	93.1	89.7	85.5
% of nominal concentration	105.5	99.4	93.3	96.8
Imprecision [%CV]	2.1	6.2	2.4	10.4

Extracted sample stability at +4°C, 72 h

QC level [ng/mL]	2	4	20	40
	96.5	112	96.4	104
	101	95.3	103	95.2
	94.2	105	93.5	99.5
	100	96.9	92.6	89.3
% of nominal concentration	97.9	102.3	96.4	97.0
Imprecision [%CV]	3.1	7.7	4.7	6.3

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number
Reference Materials		
Tacrolimus	U.S. Pharmacopeial Convention	1642802
D2,13C-Tacrolimus	Toronto Research Chemicals Inc.	F370002
Test Materials		
Red blood cells	University of Colorado Hospital	W20091305500 V
Plasma	University of Colorado Hospital	W2017130556300Q
Solvents		
Acetone CHROMASOLV, HPLC, ≥ 99,9 %	Sigma-Aldrich	439126-4 L
Acetonitrile Optima LC/ MS, UHPLC- UV	Thermo Fisher Scientific	A955-4
Isopropanol 99.9 %, HPLC	Fisher Scientific	BP2632-4
Methanol Optima LC/ MS	Thermo Fisher Scientific	A452-4
Water Optima LC/ MS, UHPLC- UV	Thermo Fisher Scientific	W6-4
Other Chemicals		
Formic acid	Thermo Fisher Scientific	A118P-500
Phosphate-buffered saline (PBS)	Sigma-Aldrich	D8537
Zinc sulfate	Thermo Fisher Scientific	Z68-500
Laboratory Instruments and Consumables		
0.5 – 10 µl pipet, VoluMate LIQUISYSTEMS	Mettler Toledo	17008649
1,5 mL- Eppendorf tube	Thermo Fisher Scientific	02-682-550
10 – 100 µL pipet, VoluMate LIQUISYSTEMS	Mettler Toledo	17008651
10 µL- pipet tips with filter, sterile	Neptune	BT 10XLS3

100 – 1000 µl pipet, VoluMate LIQUISYSTEMS	Mettler Toledo	17008653
100 µL- pipet tips with filter, sterile	Neptune	BT 100
1000 µL- pipet tips with filter, sterile	Multimax	2940
2 – 20 µL pipet, VoluMate LIQUISYSTEMS	Mettler Toledo	17008650
2 mL- Eppendorf tube	Thermo Fisher Scientific	02-681-258
20 – 200 µL pipet, VoluMate LIQUISYSTEMS	Mettler Toledo	17008652
20 µL- pipet tips with filter, sterile	GeneMate	P-1237-20
200 µL- pipet tips with filter	Multimax	2938T
200 µL- pipet tips with filter, sterile	Multimax	2936J
50 mL- Falcon tube	BD Falcon	352070
300 µL inserts for HPLC vials	Phenomenex	ARO-9973-13
Balance PR2002	Mettler Toledo	1117050723
Balances AX205 Delta Range	Mettler Toledo	1119343379
Bullet Blender Homogenizer	Next Advance	BBX24
Centrifuge Biofuge Fresco	Heraeus	290395
Disposable Wipes	PDI	Q55172
Glass v ials, 4 mL	Thermo Fisher Scientific	14-955-334
Glass vials, 20 mL	Thermo Fisher Scientific	B7800-20
Gloves, nitrile	Titan Brand Gloves	44-100S
HPLC vials, 9 mm, 2 mL, clear	Phenomenex	ARO- 9921-13
Lids for HPLC vials	Phenomenex	ARO- 8952-13-B
Needle, 18G 1.5	Precision Glide	305196
Rack for Eppendorf tubes	Thermo Fisher Scientific	03-448-11
Rack for HPLC Vials	Thermo Fisher Scientific	05-541-29
Steel beads 0.9 – 2 mm	Next Advance	SSB14B
Storage boxes for freezers / refrigerators	Thermo Fisher Scientific	03-395-464
Standard multi-tube vortexer	VWR Scientific Products	658816-115
Whatman Paper, 903 Protein Saver US 100/PK	GE Whatman	2016-05

HPLC Equipment and Columns

Autosampler	CTC PAL	PAL.HTCABIx1

Binary pump, Agilent 1260 Infinity	Agilent Technologies	1260 G1312B
Binary pump, Agilent 1290 Infinity	Agilent Technologies	1290 G4220A
Micro vacuum degasser, Agilent 1260	Agilent Technologies	1260 G13798
Column oven, Agilent 1290 with 2 position	Agilent Technologies	1290 G1216C
Thermostated column compartment with integrated 6 port switching valve	Agilent Technologies	1290 G1316C
HPLC pre-column cartridge, Zorbax XDB C8 (5 µm particle size), 4.6 · 12.5 mm	Phenomenex	820950-926
HPLC analytical column, Zorbax Eclipse-XDB-C8 (5 µm particle size), 4.6 · 150 mm	Phenomenex	993967-906
Tandem Mass Spectrometer		
API5000 MS/MS with TurboIonspray source	AB Sciex	4364257
Mass spectrometry software	AB Sciex	Analyst 1.5.1

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Author(s): Shokati T, Bodenberger, N, Vinks AA, Jiang W, Alloway RR, Christians U

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Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

March 29, 2015

Re.: Our manuscript JoVE52424R3 entitled “Quantification of the Immunosuppressant Tacrolimus on Dried Blood Spots Using LC-MS/MS.”

Dr. Nguyen:

Please find the revision of our above-mentioned manuscript attached.

Below you will find a list of changes and our response to the reviewers’ comments.

Respectfully,

Uwe Christians, MD, PhD
Professor and Laboratory Director
Facharzt für Pharmakologie und Toxikologie
Facharzt für Klinische Pharmakologie
Diplomate, American Board of Clinical Pharmacology
Master in Research Quality Assurance (BARQA)

List of Changes

Editor's comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
DONE.

2. Please reduce the repetition between the Short and Long Abstract.
DONE.

3. How are the ions detected in step 3.6?
THIS INFORMATION WAS ADDED TO 3.5.

4. Please provide conditions for steps 2.6 and 2.7. (RPM, speed setting, etc.)
DONE.

5. Step 3.7 is an action step and not a note. Please revise.
THE ACTION INFORMATION IN 3.7. WAS MOVED TO 3.5. AS THIS IS MORE IN LINE WITH THE WORKFLOW.

6. Step 4.1 needs more detail or a reference.
THE COMPLETE SECTION 4 WAS REWRITTEN.

Reviewers' comments:

Reviewer #1:
Major Concerns:
N/A

Minor Concerns:
1. The authors must compare the merits of their methods with those reported in literature.
THIS IS DISCUSSED IN DETAIL IN THE PARAGRAPH STARTING ON LINE 583. IT IS IMPORTANT TO NOTE THAT NONE OF THE NUMEROUS TACROLIMUS DRIED BLOOD SPOT ASSAYS DESCRIBED IN THE LITERATURE WAS TRUELY NOVEL AS MOST OF THESE WERE VARIATIONS OF TACROLIMUS WHOLE BLOOD ASSAYS. AS OUTLINED IN SAID PARAGRAPH, THE PRESENT METHOD UNIQUELY COMBINES SAMPLE PROCESSING STRATEGIES RESULTING IN A RELIABLE ROUTINE ASSAY, WHICH HAS SUCCESSFULLY BEEN APPLIED TO THE ANALYSIS OF MORE 5000 DRIED BLOOD SPOT SAMPLES WITHOUT LOOSING A SINGLE SAMPLE.

2. Tables for intra-day and inter-day accuracy and precision and stability can be condensed by presenting mean values at different QC values.
WE HAVE DISCUSSED THIS SUGGESTION AMONG THE AUTHORS AND FELT THAT IT WILL BE IMPORTANT TO REPORT INDIVIDUAL RESULTS IN COMBINATION WITH THE APPROPRIATE DISTRIBUTION STATISTICS ANALYSIS. THE RATIONALE IS THAT THE GOAL OF THIS PUBLICATION AND ITS

SUPPORTING VIDEO COMPONENT IS TO ENABLE THE READER TO REPRODUCE OUR ASSAY. IN OUR OPINION THIS PUBLICATION PLATFORM IS A UNIQUE OPPORTUNITY TO GIVE THE INTERESTED READER A FEEL FOR WHAT THE EXPERIMENTS LOOK LIKE IN REAL LIFE AND WHAT DATA TO EXPECT, INCLUDING OUTLIERS AND FAILED ANALYSES. THIS IS IMPORTANT INFORMATION THAT TENDS TO GET LOST IN A DISTRIBUTION STATISTICAL ANALYSIS WITHOUT ACCESS TO THE RAWDATA. WE HOPE THAT THIS POSITION IS ACCEPTABLE.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
In their manuscript, Shokati and colleagues describe the development and validation of an LC-MS/MS method for the quantification of tacrolimus in dried blood spots. Overall, the article is well written and the experiments are clearly described. Although in general the experiments appear well-performed, there are several important issues that this reviewer would like to have addressed. An experiment to better address matrix effects is required and more extensive discussion of and comparison with existing literature is required.

Major Concerns:
Lines 141 & 515:The authors state that coagulation takes place in a dried blood spot. However, the reviewer is not aware of evidence that coagulation does take place (even when blood is directly applied from a fingerprick). Either the reviewers provide a reference demonstrating that coagulation does take place in filter paper, or they replace "coagulated" by "dried". Moreover, in the authors' experiments, EDTA blood was used, which does not coagulate.
ALTHOUGH TO THE BEST OF OUR KNOWLEDGE THIS STATEMENT IS CORRECT (BLOOD WITHOUT ANTICOAGULANT SUCH AS A CAPILLARY BLOOD DROP AFTER FINGER STICK WILL AT LEAST PARTIALLY COAGULATE), WE HAVE COMPLIED WITH THE REVIEWER'S REQUEST AND DELETED THE STATEMENTS TO THIS EFFECT FROM THE INTRODUCTION AND DISCUSSION SECTIONS.

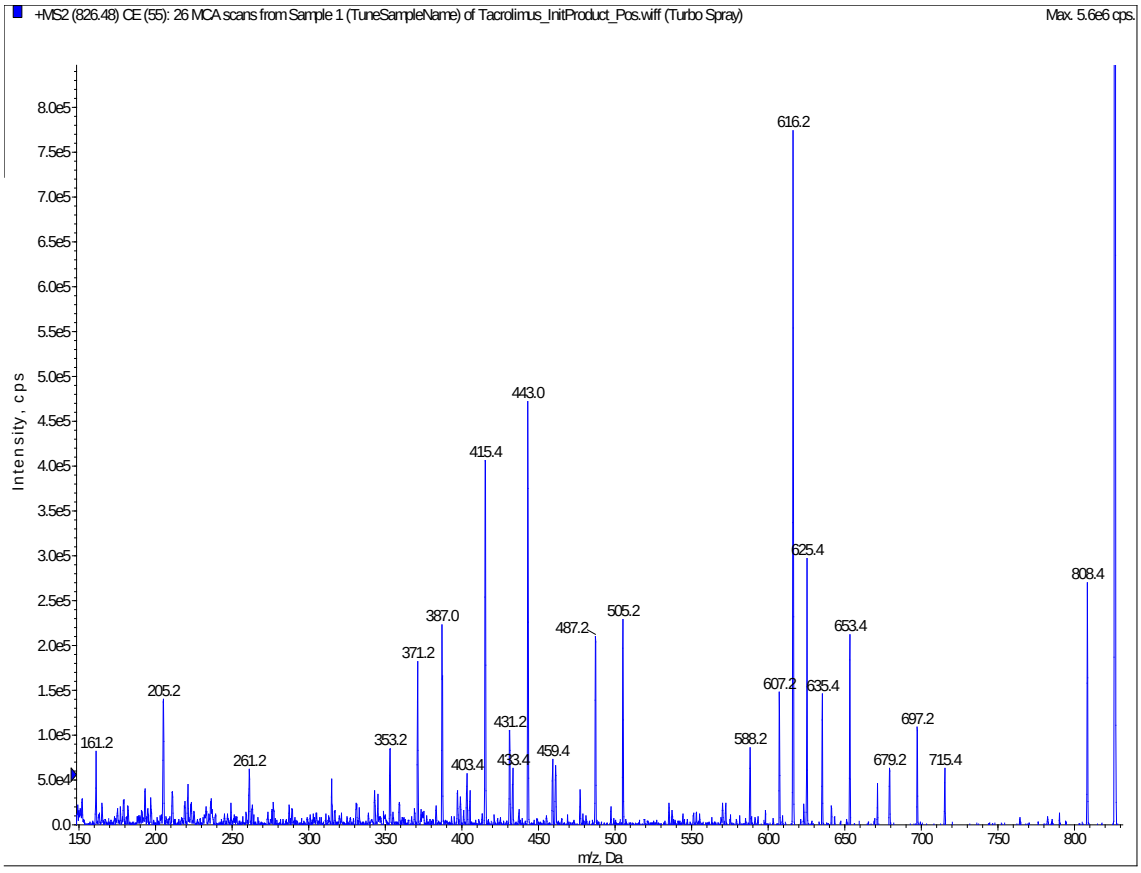
Line 181-183: The authors have in the end 10% methanol in blood. Organic solvent should not exceed 5%. The authors need to mention that this 10% is not optimal.
THIS STATEMENT WAS POTENTIALLY MISLEADING. AS COMMON, CALIBRATORS AND QUALITY CONTROL SAMPLES WERE PREPARED IN BULK AND AFTER SPIKING AND INCUBATION AT 37°C UNDER GENTLE SHAKING, SAMPLES WERE ALIQUOTED INTO 1.5 ML POLYPROPYLENE TUBES WITH CONICAL BOTTOMS AND SNAP-ON LIDS. THE PARAGRAPH IN THE TEXT HAS BEEN RE-WRITTEN.

Line 203: There is an issue with references, that became apparent here. Refs 44,45 should be 43,44. Also ref 45 (line 208) is not correct; also other references are not correct (e.g. there is no ref 49 in the ref list, while it is referred to on line 585)
WAS CORRECTED.

Line 217-219: The amount of IS is rather high. Calculations by this reviewer indicate that it likely corresponds to the concentration of the highest calibration standard. The authors

should motivate the choice for this high concentration.
AN INTERNAL STANDARD CONCENTRATION IN THE UPPER CONCENTRATION RANGE OF THE CALIBRATION CURVE WAS INTENTIONALLY CHOSEN TO MINIMIZE VARIABILITY OF THE INTERNAL STANDARD PEAK CAUSED BY RANDOM BASELINE SHIFTS AND NOISE. PLEASE NOTE THAT ESPECAILLY ERRORS AT THE PEAK BASE (EG BY THE INTEGRATION SOFTWARE INCLUDING A BASELINE SHIFTS IN THE INTERNAL STANDARD'S PEAK AREA) HAS A MARKED EFFECT ON THE AREA UNDER THE PEAK. THIS IS AN AUTOMATED HIGH-THROUGHPUT ASSAY USED FOR FDA STUDIES AND MANUAL RE-INTEGRATION IS NOT ALLOWED. THEREFORE IT WAS IMPERATIVE TO ENSURE THAT INTEGRATION BY THE SOFTWARE WAS EASILY REPRODUCIBLE AND THE INFLUENCE OF INTEGRATION ERRORS WAS MINIMIZED.

Line 249-251: are the mass transitions correct? A typical transition for tacrolimus in literature appears to be 821-768. The authors have to elaborate on this. Also the choice for a sodium adduct should be elaborated upon.
THE ION TRANSITIONS ARE CORRECT. FRAGMENTATION PATTERNS DEPEND ON MANY FACTORS INCLUDING, BUT NOT LIMITED TO INSTRUMENT DESIGN, COLLISION GAS AND COLLISION VOLTAGES. FOR THE REVIEWER'S INFORMATION, WE PROVIDE A REPRESENTATIVE MS/MS SPECTRUM RECORDED ON ONE OF THE INSTRUMENTS RUNNING THE PRESENT ASSAY:



THE LC-MS/MS INSTRUMENTS IN OUR LABORATORY ENVIRONMENT HAVE ALWAYS PRODUCED SODIUM ADDUCTS EVEN IF OTHER IONS WERE ADDED EG TO FACILITATE THE FORMATION OF AMMONIUM ADDUCTS. IN OUR

LABORATORY SODIUM ADDUCTS HAVE ALWAYS GIVEN THE BEST RESULTS. WE ACKNOWLEDGE THAT THIS MAY BE DIFFERENT IN OTHER LABORATORIES. NEVERTHELESS, BASED ON THE LITERATURE, THE RELIANCE ON SODIUM ADDUCTS FOR THE QUANTIFICATION OF TACROLIMUS AND OTHER IMMUNOSUPPRESSANTS IS NOT UNCOMMON.

Line 262: quadratic fit is sometimes used for very wide calibration ranges. However, the calibration range here is not wide (only 1-50 ng/ml). The authors should explain their choice for this model. Did they calculate residuals and used the lowest sum of residuals?
IN OUR EXPERIENCE A QUADRATIC FIT IN COMBINATION WITH AN MS/MS DETECTOR WILL ALWAYS GIVE MORE ACCURATE RESULTS AT THE LOWER AND UPPER LIMITS OF QUANTIFICATION THAN LINEAR FITS. THIS WAS ALSO THE CASE WITH PRESENT ASSAY. BEFORE THE DECISION FOR UTILIZING A QUADRATIC FIT MADE, SEVERAL WEIGHTING AND CURVE FITTING ALGORITHMS WERE TESTED AND THE QUADRATIC FIT, AS USUAL, GAVE THE BEST PRECISION AND ACCURACY AT THE ENDS OF THE CALIBRATION CURVE.

One of the prime advantages that the authors put forward is lack of matrix effects. However, they have only examined matrix effects in a very rough way. It is essential that the authors perform the classical 'Matuszewski experiment', in which 3 series of sampled are compared: neat standards, extracts of DBS spiked prior to extraction and extracts of blank DBS spiked post extraction. This is an essential comment.
WE RESPECT THE REVIEWER'S CONFIDENCE IN THE MATUSZEWSKI EXPERIMENT. NEVERTHELESS, A POST-INFUSION EXPERIMENT CANNOT BE CONSIDERED "ROUGH" AND IS A VALID AND ACCEPTED APPROACH. PLEASE NOTE THAT THE MATUSZEWSKI EXPERIMENT IS ONLY A MOMENTARY SNAP-SHOT AS IT IS 'BLIND' TO POTENTIAL MATRIX EEFECTS THAT ARE DANGEROUSLY CLOSE TO THE ANALYTE PEAK, BUT DO NOT HAVE AN EFFECT YET, ESPECIALLY WHEN A RATHER NEW HPLC COLUMN IS USED AS USUALLY THE CASE DURING PRE-STUDY ASSAY VALIDATION. AS DURING ROUTINE MEASUREMENT SEPARATION POWER OF A COLUMN DECREASES, THERE IS THE RISK THAT AT SOME POINT A PREVISOULY UNNOTICED MATRIX EFFECT CLOSE TO THE ANALYTE PEAKS BECOMES A PROBLEM. IN CONTRAST TO THE MATUSZEWSKI EXPERIMENT, A POST-INFUSION EXPERIMENT WILL SHOW MATRIX EFFECTS OVER THE COMPLETE ANALYSIS PERIOD AND WILL GIVE INFORMATION ON HOW WELL SEPARATED FROM POTENTIAL MATRIX EFFECTS THE PEAKS OF INTEREST ARE. AS THE REVIEWER MAY ALREADY HAVE GUESSED BASED ON THE FACT THAT WE STUDIED EXTRACTION RECOVERY USING A MATUSZEWSKI EXPERIMENT, WE ALSO DETERMINED THE MATRIX EFFECTS DURING THIS EXPERIMENT. THESE WERE WITHIN ACCEPTABLE LIMITS (WITHIN 89.5-105.3% OF THE DETECTOR RESPONSE OF CORRESPONDING DRUG AMOUNTS IN NEAT STANDARD SOLUTIONS). AS IN OUR EXPERIENCE THE POST-INFUSION EXPERIMENT TO ASSESS THE PRESENCE AND RISK OF MATRIX EFFECTS IS OF MORE RELEVANCE FOR THE ROBUSTNESS OF AN ASSAY INTENDED FOR HEAVY ROUTINE USE, WE DECIDED TO FOCUS ON THOSE RESULTS. WE HOPE THAT THIS WILL BE ACCEPTABLE.

Another essential comment is that the authors did not evaluate accuracy & precision at LLOQ. Strictly taken, they cannot consider 1 ng/ml as the LLOQ, as they state themselves on line 271-272 that for LLOQ bias and imprecision should be less than

20%. However, no QC at LLOQ level was included, hence no true statement can be made that 1 ng/ml is a true LLOQ. This is a major shortcoming. However, looking at the available data for the 2 ng/ml level, this reviewer is convinced that the methodology will likely be OK for 1 ng/ml as well. Still, this shortcoming has to be clearly mentioned in the text (cfr on line 566 is it clearly mentioned that the LLOQ is 1 ng/ml).
ACCURACY AND PRECSION AT THE LLOQ WAS EXTENSIVELY TESTED. BASED ON CURRENT FDA GUIDANCE, THE LLOQ CAN BE DETERMINED BY BACK-EXPTRAPOLATION OF THE CALIBRATORS AND THERE IS NO REQUIREMENT FOR A QC AT THE LLOQ. IN FACT, FDA GUIDANCE RECOMMENDS THE LOWEST QC TO BE 3X THE LLOQ. PLEASE NOTE THAT WE COULD NOT REPORT ALL RESULTS IN DETAIL TO KEEP THE MANUSCRIPT WITHIN REASONABLE SPACE LIMITS. THE ACCURACY AT THE LLOQ WAS 101.3% AND THE PRECISON WAS 6.3 (MEASURED OVER 20 DAYS, N=2/ DAY, N=40 TOTAL). FOR THE REVIEWER'S REFERENCE WE HAVE INCLUDED THE RELEVANT RESULT TABLE IN THE APPENDIX TO THIS RESPONSE (COPIED FROM THE VALIDATION REPORT AS SUBMITTED TO THE FDA).

Cross-talk between tacrolimus and its internal standard needs to be tested: is the signal for tacrolimus blank when only IS is present (and vice versa)?
THIS WAS TESTED (EG BY INCLUSION OF ZERO SAMPLES IN THE VALIDATION AND RUN BEFORE EACH OF OUR CALIBRATION CURVES) AND NONE WAS FOUND (NONE= <15% OF THE TACROLIMUS SIGNAL AT THE LLOQ). THIS WAS CRITICAL FOR OUR DECISION TO USE THE INTERNAL STANDARD AT A CONCENTRATION AT THE HIGHER END OF THE CALIBRATION CURVE.

Overall, there should be more extensive referral and comparison with existing methods. What is precisely the added-value of the presented method? As stated above, the authors suggest that their clean-up is better than that of other published methods, although they have failed to examine matrix effects in detail. Again, this is an experiment that needs to be performed.
THE COMPARISON WITH OTHER PUBLISHED ASSAYS IS DISCUSSED IN THE PARAGRAPH STARTING ON LINE 583. PLEASE NOTE THAT DUE TO THE REALTIVELY LARGE NUMBER OF PUBLISHED TACROLIMUS DRIED BLOOD SPOT LC-MS/MS ASSAYS, WE COULD NOT POINT OUT DIFFERENCES TO EACH INDIVIDUAL ASSAY AND RATHER WERE LIMITED TO DISCUSSING THE OTHER PUBLISHED ASSAYS IN AGGREGATE. IN TERMS OF OUR PRESUMED FAILURE TO APPROPRIATELY EVALUATE MATRIX EFFECT, WE WOULD LIKE TO REFER TO THE RELEVANT DISCUSSIONS ABOVE AND EMPHASIZE THAT WE USED BOTH POST-COLUMN INFUSION AND MATUSZEWSKI APPROACHES AND THAT BOTH CONISTENTLY INDICATED THE LACK OF RELEVANT MATRIX EFFECTS. DUE TO ITS GREATER RELEVANCE, WE DECIDED TO RELY HERE MAINLY ON THE POST-INFUSION EXPERIMENTS AS DESCRIBED BY MÜLLER ET AL. [REFERENCE 45].

Figure 3A: in fact, when this is a blank that is handled like any other sample, the signal of IS should be here. This reviewer understands that inclusion of the IS would not make it clear anymore to see the (low) background signal. Still, it has to be mentioned that this sample also lacks IS.
PLEASE NOTE THAT APPLICABLE FDA GUIDANCE DISTINGUISHES BETWEEN 'BLANK' AND 'ZERO' SAMPLES. A BLANK SAMPLE IS A TRUE BLANK WITHOUT ANALYTE AND INTERNAL STANDARD, WHILE A ZERO SAMPLE DOES NOT

CONTAIN ANALYTE, BUT IS SPIKED WITH THE INTERNAL STANDARD. THUS, THE LEGEND TO FIGURE 3A IS CORRECT AND A BLANK IS SHOWN. AS CORRECTYLY NOTED BY THE REVIEWER, WE DECIDED TO SHOW A BLANK INSTEAD OF A ZERO SAMPLE TO SHOW THAT THERE WAS NEITHER MATRIX INTERFERENCE WITH THE ANALYTE NOR WITH THE INTERNAL STANDARD PEAK.

Minor Concerns:
Line 96-98 about physicochemical characteristics of tacrolimus is no essential information; this can be removed
WE RESPECTFULLY DISAGREE WITH THE REVIEWER. THE PHYSICOCHEMICAL PROPERTIES PROVIDE THE BASIS FOR THE EXTRACTION PROCEDURE AND HPLC CONDITIONS AND WE WOULD PREFER TO KEEP THIS INFORMATION IN THE MANSUCRIPT.

line 127: 50 ul is NOT typically collected from a fingertip. The reviewer refers to line 133, where it is stated that DBS are typically 20 ul. In this reviewer's experience, 20-30 ul is indeed typically collected.
THIS STATEMENT WAS MISLEADING. WHAT THIS STATEMENT WAS SUPPOSED TO REFER TO WAS THAT FILLING THE CIRCLE ON THE WHATMAN 903 PAPER IS EQUIVALENT TO 50 µL OF BLOOD. WE HAVE DELETED THIS STATEMENT FROM THE INTRODUCTION AND HAVE ADDED A CORRECTED STATEMENT STARTING ON LINE 537 OF THE REVISED MANUSCRIPT. WE HAVE ALSO ADDED REFERENCE [46].

Line 223: m n should be min
CORRECTED

Line 416: insert "calibrators" after the brackets
DONE.

Line 588: replace "have" by "has"

APPENDIX

Table 8.1-2: Intra- and Inter-day Accuracy and Precision of Calibrators (CCs)

Intra-Day Accuracy and Precision DBS

Day 1 - 20 CCs

Validation Day	1.0	2.5	5.0	10.0	25.0	50.0	R^2
Day 1	101.0	104.0	91.5	93.6	111.0	102.0	0.9982
	107.0	107.0	91.9	99.0	105.0	105.0	
	86.9	95.1	102.0	88.4	96.1	99.2	
	89.3	95.8	106.0	88.1	100.0	95.2	
	108.0	110.0	103.0	95.4	105.0	97.2	
	121.0	100.0	95.4	102.0	108.0	95.0	
Intra-day Accuracy	102.2	102.0	98.3	94.4	104.2	98.9	
Intra-Day Precision	12.7	6.1	6.2	5.6	5.4	4.0	
Day 2	94.4	101.0	95.5	94.4	102.0	102.0	0.9994
	98.8	106.0	110.0	95.7	101.0	98.0	
Intra-day Accuracy	96.6	103.5	102.8	95.1	101.5	100.0	
Intra-Day Precision	3.1	3.5	10.3	0.9	0.7	2.8	
Day 3	97.9	101.0	96.2	102.0	106.0	106.0	0.9990
	109.0	98.8	94.8	98.4	97.6	93.4	
Intra-day Accuracy	103.5	99.9	95.5	100.2	101.8	99.7	
Intra-Day Precision	7.8	1.6	1.0	2.5	5.9	8.9	
Day 4	104.0	102.0	96.1	94.4	108.0	96.6	0.9989
	106.0	93.8	103.0	94.0	100.0	102.0	
Intra-day Accuracy	105.0	97.9	99.6	94.2	104.0	99.3	
Intra-Day Precision	1.4	5.8	4.9	0.3	5.7	3.8	
Day 5	97.4	101.0	94.1	95.5	104.0	97.2	0.9994
	108.0	104.0	97.6	95.6	104.0	101.0	
Intra-day Accuracy	102.7	102.5	95.9	95.6	104.0	99.1	
Intra-Day Precision	7.5	2.1	2.5	0.1	0.0	2.7	
Day 6	113.0	93.6	97.8	101.0	107.0	90.0	0.9967
	113.0	94.9	87.2	90.3	105.0	107.0	
Intra-day Accuracy	113.0	94.3	92.5	95.7	106.0	98.5	
Intra-Day Precision	0.0	0.9	7.5	7.6	1.4	12.0	
Day 7	101.0	95.4	102.0	97.6	102.0	103.0	0.9996
	100.0	103.0	97.1	105.0	96.8	97.5	
Intra-day Accuracy	100.5	99.2	99.6	101.3	99.4	100.3	

Intra-Day Precision	0.7	5.4	3.5	5.2	3.7	3.9	
Day 8	106.0	102.0	103.0	N/D	99.5	105.0	0.9994
	87.9	105.0	95.8	102.0	98.1	95.8	
Intra-day Accuracy	97.0	103.5	99.4	102.0	98.8	100.4	
Intra-Day Precision	12.8	2.1	5.1		1.0	6.5	
Day 9	113.0	96.8	104.0	101.0	106.0	103.0	0.9992
	97.6	94.3	93.9	96.0	98.6	95.8	
Intra-day Accuracy	105.3	95.6	99.0	98.5	102.3	99.4	
Intra-Day Precision	10.9	1.8	7.1	3.5	5.2	5.1	
Day 10	108.0	103.0	94.1	92.0	108.0	98.3	0.9987
	101.0	103.0	94.4	95.2	104.0	98.4	
Intra-day Accuracy	104.5	103.0	94.3	93.6	106.0	98.4	
Intra-Day Precision	4.9	0.0	0.2	2.3	2.8	0.1	
Day 11	93.5	108.0	97.6	90.8	100.0	98.8	0.9993
	94.6	109.0	106.0	97.8	104.0	100.0	
Intra-day Accuracy	94.1	108.5	101.8	94.3	102.0	99.4	
Intra-Day Precision	0.8	0.7	5.9	4.9	2.8	0.8	
Day 12	108.0	101.0	Failed	106.0	103.0	100.0	0.9990
	99.8	91.4	91.2	105.0	93.3	101.0	
Intra-day Accuracy	103.9	96.2	91.2	105.5	98.2	100.5	
Intra-Day Precision	5.8	6.8		0.7	6.9	0.7	
Day 13	103.0	90.3	97.0	101.0	101.0	105.0	0.9987
	109.0	Failed	104.0	91.8	105.0	93.3	
Intra-day Accuracy	106.0	90.3	100.5	96.4	103.0	99.2	
Intra-Day Precision	4.2		4.9	6.5	2.8	8.3	
Day 14	99.1	108.0	102.0	93.8	104.0	94.7	0.9990
	98.2	99.0	101.0	93.8	103.0	104.0	
Intra-day Accuracy	98.7	103.5	101.5	93.8	103.5	99.4	
Intra-Day Precision	0.6	6.4	0.7	0.0	0.7	6.6	
Day 15	96.5	99.3	114.0	94.0	97.8	97.5	0.9992
	93.8	103.0	98.7	101.0	100.0	103.0	
Intra-day Accuracy	95.2	101.2	106.4	97.5	98.9	100.3	
Intra-Day Precision	1.9	2.6	10.8	4.9	1.6	3.9	
Day 16	100.0	108.0	103.0	99.1	98.9	106.0	0.9991
	102.0	92.1	92.2	103.0	101.0	94.2	
Intra-day Accuracy	101.0	100.1	97.6	101.1	100.0	100.1	

Intra-Day Precision	1.4	11.2	7.6	2.8	1.5	8.3	
Day 17	96.0	105.0	100.0	96.0	102.0	97.7	0.9996
	107.0	99.6	94.4	97.9	103.0	101.0	
Intra-day Accuracy	101.5	102.3	97.2	97.0	102.5	99.4	
Intra-Day Precision	7.8	3.8	4.0	1.3	0.7	2.3	
Day 18	99.7	104.0	98.1	94.7	106.0	101.0	0.9994
	107.0	96.1	95.4	100.0	100.0	97.4	
Intra-day Accuracy	103.4	100.1	96.8	97.4	103.0	99.2	
Intra-Day Precision	5.2	5.6	1.9	3.7	4.2	2.5	
Day 19	97.4	99.0	92.3	93.2	108.0	96.4	0.9985
	111.0	110.0	94.1	94.2	104.0	101.0	
Intra-day Accuracy	104.2	104.5	93.2	93.7	106.0	98.7	
Intra-Day Precision	9.6	7.8	1.3	0.7	2.8	3.3	
Day 20	93.7	104.0	97.9	94.8	101.0	97.5	0.9994
	98.4	107.0	107.0	94.5	103.0	102.0	
Intra-day Accuracy	96.1	105.5	102.5	94.7	102.0	99.8	
Intra-Day Precision	3.3	2.1	6.4	0.2	1.4	3.2	
Inter-Day Accuracy and Precision DBS							
Inter-day Accuracy	**101.3**	**101.0**	**98.4**	**96.7**	**102.5**	**99.4**	AVERAGE
Inter Day Precision	**6.7**	**5.3**	**5.5**	**4.4**	**3.7**	**3.9**	**0.9990**

- *N/D means no peaks were detected. This may be most likely due to wrong sample preparation or failure during sample injection.*
- *Those samples labeled as failed did show very high levels of tacrolimus which may be due to contamination with higher stock solution during sample preparation or a pipetting error of the internal standard solution. Those values were not taken into consideration for statistical calculation.*
- *Those values labeled as red did not pass the acceptance criteria. However, those values were included in the distribution statistical calculation.*